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An Investigation of Mortality of the Pearl Oyster, *Pinctada maxima*, in Western Australia.

BY

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AND

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PERTH
WESTERN AUSTRALIA

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Fisheries Department

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PERTH

REPORT

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AN INVESTIGATION OF MORTALITY OF THE PEARL OYSTER, *Pinctada maxima*, IN WESTERN AUSTRALIA

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AN INVESTIGATION OF MORTALITY OF THE PEARL OYSTER, *Pinctada maxima*, IN WESTERN AUSTRALIA

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ABSTRACT

A three year investigation into the cause of abnormally high mortality rates of the pearl oyster, Pinctada maxima, on the cultured pearl industry's lease sites in the North West of Western Australia was completed on 30 June 1983. The objective of this study was to determine environmental, physiological and pathological factors predisposing farm oysters to excessive levels of mortality and, if possible, make recommendations to alleviate this problem.

As mass mortalities may have complex causes, complementary field and laboratory studies were instigated. The culture industry's methods of collection, transportation and farm husbandry were examined. Environmental parameters were monitored. Various empirical treatments were made on test lots of oysters to find out the effects on oyster mortality of factors such as the seasonal period during which they were transported to the lease site after collection, and the depth and density conditions under which they were then maintained. Laboratory research included comparisons of the physiological condition of oysters kept under different temperature regimes as well as pathological procedures aimed at identifying causative disease agents.

Research was primarily carried out on pearl oysters from the largest company which was experiencing the greatest losses. Their mortality problem was shown to follow transportation of oysters in high densities on vessels with inadequate water circulation which resulted in a massive build-up of bacteria in the carrier tanks. The main infectious agents were identified as marine Vibrio bacteria.

Water temperature was shown to be the environmental factor most closely associated with mortality. The greatest incidence of mortality occurred during winter. It was therefore postulated that bacterial invasion occurred in many oysters, weakened by exposure to low annual ambient temperatures, when subjected to high concentrations of bacteria during transportation.

The company was recommended to improve the circulation of water within their carrier tanks, to carry as few oysters per trip as economically possible and to minimize the transportation of oysters during the winter months.

I INTRODUCTION

The Western Australian pearling industry is Australia's fourth most important fishing industry. Pearl production is based on the utilization of naturally grown oysters that are removed from their natural beds and then transported to lease sites where pearl formation is induced by artificial means. An excessive mortality of pearl oysters following removal from the natural beds has had a serious impact upon the industry since 1974. Losses on the lease sites can amount to 80% of the harvested shell, although losses of 30-60% are more common. These rates contrast markedly with accepted "normal" losses of 10-20%. In addition to the losses, an equally significant factor is that affected oysters that do not die but recover, develop deformed nacre and are useless for half-pearl or mother-of-pearl production.

Excess mortality has two major effects on the industry. Firstly, the obvious effects are a direct financial loss to operators associated with the cost of collecting wild oysters together with consequent lost production of pearls over the ensuing seasons. Secondly, excessive mortality also forces operators to remove more oysters than they would require if mortality was not present and as the industry is based on natural stocks the mortality problem places increasing pressure from fishing on the natural population.

The aim of the investigations reported in this paper was to determine the factors and possible causes associated with mortality*. Most of this work was done in conjunction with the largest company which was experiencing the greatest and most persistent mortality problem. Incidental outbreaks of oyster mortality on other lease sites may have other determining factors involved.

II THE WESTERN AUSTRALIAN PEARL CULTURE INDUSTRY

For more than a century pearl and pearl shell fishing has been a valuable industry along the Northern coast of Western Australia. For most of its colourful history, the industry revolved around the collection of shell or mother-of-pearl, rather than the natural pearls themselves (Bain, 1982). The mother-of-pearl shell from the silver or gold-lipped pearl oyster, *Pinctada maxima* (Jameson), was used for button manufacture, and any natural pearls found were a bonus. Broome became the major pearling centre; and by the early 1900's it had a fleet of more than 300 sailing vessels, called luggers, fishing for shell.

The industry survived severe disruption during World War II only to find the demand for pearl shell dwindled to negligible proportions following the advent of plastic as a material for

* The purpose of this paper is to summarize the principal findings of the mortality study, aspects of which will be reported on in detail elsewhere (Pass and Dybdahl, in prep).

buttons. Interest in pearling was again revived in 1955 with the establishment of pearl culture farms. Techniques for artificially stimulating pearl production were developed in Japan early this century, and a joint Japanese-Australian firm, Pearls Pty Ltd (PPL), produced Australia's first harvest of cultured pearls from their farm lease at Kuri Bay north of Broome in 1957. Licences to culture pearls have since been issued to eight other Western Australian companies for lease sites in King Sound, Beagle Bay, Willie Creek, Roebuck Bay, the Monte Bello Islands and Shark Bay, as well as a Northern Territory venture which collects its shell from waters off Western Australia. This industry has grown until in 1982 it employed some 270 people and produced in excess of $18 million worth of cultured pearls and shells annually. This made it Western Australia's second most valuable fishing industry after rock lobsters. A more detailed account of Western Australia's pearling industry is to be given elsewhere. The description given here is intended only to provide the background essential to understanding the mortality studies.

The pearl culture techniques employed in Australia are similar to those described for pearl farming in Japan by Mizumoto (1979). The procedures are briefly as follows: the collection and acclimatization of the host animal, the operation to insert the nucleus, a period of convalescence followed by a further period of maintaining the oyster during cultivation, before harvest of the pearl products.

As the culture of pearls is more of an art than a science, the individual companies have evolved many variations of the general procedures given above. For example, some companies maintain their oysters directly on the sea bottom, while others hold them in baskets suspended from log rafts or long-lines.

While some pearl oyster deaths are expected to occur as a result of the operational insertion of the nucleus and natural mortality may occur during the several years during which operated oysters must be maintained before pearls are harvested, the mass mortality of oysters leading to this study was noticed to occur between the time of collection and transport of the oysters to the lease site and the operation itself i.e. during the acclimatization period before operation. Since the largest company, Pearls Proprietary Ltd has experienced a continuing mortality problem since 1974, their methods of collection, transportation and method of holding oysters will be given as an example.* Further details on the rest of their culture techniques, such as the nucleus insertion operation are given in Hancock (1973).

Along with most of the other companies, shell for PPL are fished in the waters off Eighty Mile Beach south of Broome (Fig. 1). The fishing season is usually limited to between March and November as the pearling fleet is laid-up during the more cyclone-prone summer months.

*This description of Pearls Proprietary Ltd's Kuri Bay operation refers to the period up to and including June 1983. In July 1983, PPL announced that it would not collect any more live shell and would begin to phase-out its production operations at Kuri Bay.

Shells are gathered during neap tide periods by divers using Hookah gear while being towed behind luggers. When brought on deck, these shells are cleaned of sponges and other fouling organisms with a tomahawk.

Fishing regulations designed to encourage the use of live shell for culture purposes allow only shell greater than 203 mm (8") measured dorso-ventrally from the shell valve hinge to the growth edge, to be killed for use as mother-of-pearl products.

Live shell are transferred to the larger transport ship where they are sorted into arbitrary size categories as proportionally larger nuclei will be inserted into the larger oysters when they are later operated upon. Before 1982 there were three size categories (small <102 mm; medium 102 mm to 140 mm; and large >140 mm). These size categories were adjusted slightly upwards during the 1982 season when the new minimum permissible size of shell was increased from 102 mm to 114 mm (4.5").

Oysters of similar size are placed into baskets made of plastic covered wire with dimensions of 61 cm x 23 cm x 23 cm. Each basket has nine partitions to house 10 oysters in the vertical position, i.e. hinge down. The baskets are then stacked underwater in the four carrier tanks built into the hold. These tanks have a total capacity in excess of 20 000 oysters although usually no more than 15 000 oysters are transported per trip. Seawater is circulated through these tanks when the boat is at anchor by pumping in outside water and spraying it into each tank from slits in a pipe around the upper perimeter which then displaces water through four bottom drain holes in the hull. When the ship is in motion, a venturi system on two additional holes per tank pipes water to the surface of each tank.

The oysters are transported from the fishing grounds to the Kuri Bay lease site, a distance of almost 700 km to the north (Fig. 1), which takes the ship approximately 37 hours. During the longer neap fishing periods of seven or eight days, the two transport vessels, "Merindah Pearl" and "Kuri Pearl" alternately collect oysters from the luggers and transport them to Kuri Bay to reduce the amount of time oysters are held on board to a maximum of four or five days.

Upon arrival at the lease site, the baskets of oysters aboard these transport ships are quickly unloaded onto floating wooden rafts. These rafts are constructed by lashing together pine poles to form basic units 7 m x 7 m. Unit flotation is provided by 8-10 (200 L) empty metal or foam filled rubber drums lashed under the poles. Ten units are joined in two rows of five to complete a raft. One hundred baskets are suspended about 70 cm apart on the ten parallel poles of each unit i.e. 1 000 baskets per raft. The baskets are suspended from the poles with 5-6 m lengths of 12 mm black polyethylene rope. During the year-long acclimatization period before the nucleus operation, the oysters are only disturbed approximately every three months to clean off fouling organisms from the living oysters and remove any dead oysters from the baskets.

III MATERIALS AND METHODS

1. Field studies

When trying to understand the cause of new outbreaks of oyster mass mortalities, Sindermann (1979) made the recommendation that as quickly as possible, and even before adequate research information on physiological and environmental aspects of the mortality is available, empirical attempts to find ways to alleviate effects of mortalities should be instituted. Following this general advice, manipulative field experiments with test lots of pearl oysters were conducted at the same time that background data on environmental parameters were being collected.

Excellent co-operation was given by the pearl culture industry. Field work therefore needed to be only slightly constrained by practical considerations in order to minimize disruption to managerial procedures. Recognising the considerable value of the large number of oysters involved in manipulation experiments, all oysters were returned to the lease operators at the finish of each experiment.

1.1 Area of study

Pearl oysters are collected on the fishing grounds off Eighty Mile Beach and transported to various lease sites near Roebuck Bay, King Sound and Kuri Bay (Fig. 1).

The northernmost Western Australian lease site at Kuri Bay (15°, 20' S Lat., 124°, 35' E Long) experienced most of the oyster mortality within the culture industry and consequently most of the field work was conducted at this lease site. At this lease, the culture method of holding oysters involved placing them in baskets which are suspended from floating rafts anchored in eighteen protected localities throughout this area. Most of the water quality sampling and manipulative field experimentation of this study were conducted on the raft designated by the company as Kuri Bay C Place which was conveniently located within a kilometre of the main settlement (Fig. 1).

1.2 Water quality parameters

As no published accounts are known of nearshore marine environmental parameters for the Kimberley region, a water sampling programme was instigated on the fishing grounds and oyster lease sites. For comparative purposes, parallel methodology with the W.A. Department of Conservation and Environment's more comprehensive study of the Dampier Archipelago was used. The water samples from both studies were processed by the University of Western Australia's Nutrient Analysis laboratory.

During the period from May 1981 to April 1983, a total of 36 sets of water samples were taken on an opportunistic basis while on field trips. Each set consisted of water sampled from one metre below the surface and another taken one metre above the sea bottom. Discrete water samples were collected using a plastic five litre Niskin-type bottle of local manufacture.

At each sampling, the following parameters were measured: air and water temperature, salinity, turbidity, chlorophyll a (as a measure of amount of phytoplankton), orthophosphate phosphate (PO_4-P), total phosphorus (TOT-P), ammonium nitrogen (NH_4-N), nitrite plus nitrate nitrogen (NO_2 + NO_3-N) and total Kjeldahl nitrogen (TKJN).

Temperatures were measured with a laboratory mercury thermometer (^oC); salinity checked using specific gravity with a hydrometer (g/cm^3); and turbidity expressed as a Secchi disc reading in metres.

Chlorophyll a samples were filtered from the sampling bottle on site. Approximately two litres of water were filtered through a 47 mm glass fibre filter (GF/C) under low vacuum pressure, using a Nalgene filter unit mounted on a plastic two litre graduated cylinder. The filter was air dried by increasing the vacuum, removed with forceps, folded into quarters, and stored in manilla envelopes. The water volume (to the nearest 10 ml) filtered was noted and all envelopes were stored in airtight plastic bags and frozen until required for analysis by the Nutrient Laboratory.

Water samples for nutrient analysis were decanted into whirlpacks (Namco), stored on wet ice during field sampling, and kept frozen upon return to the Nutrient Laboratory until analysis could be carried out. Details of analytical methods and the capabilities of the techniques used by the Laboratory to determine nutrient values (expressed in $\mu g/l$) are given in Chiffings (1979).

From fifteen of the above bottom samples taken between May 1981 and May 1982, an additional 100 ml water sample was decanted into small bottles and prepared as a check for hydrogen sulfide using the sulfide determination methods in Skyring et al. (1979). These analyses were done by Judi Hansen in CSIRO's Division of Oceanography at Marmion W.A.

On three different occasions during May and July 1981, the amount of dissolved oxygen in the sea water circulating around the oysters in the carrier tanks aboard the transport ships and then later under the rafts where the oysters were suspended was estimated using a D.O. meter (Delta Scientific).

1.3 Industry records

Environmental data records based on a more systematic sampling programme were obtained from biologists stationed at the Kuri Bay lease site. Water quality parameters had been measured on a bi-monthly basis during 1976 and 1977 near at least six oyster raft locations (M. Yasui,, pers. comm.). Since March 1978, water samples from the same three stations designated Kuri Bay B, S-Place, and Camden B were sampled regularly during one mid-neap tide period of the month, and stations designated Kuri Bay C, Hiro Bay and Sampson were measured during the other neap period (Fig. 1). At each station, water

quality was sampled using a Niskin-type bottle at depths of 30 cm below the surface, 5 m (the usual depth of oysters in baskets suspended from the raft) and 1 m above the bottom (the depth of water where the rafts were anchored was approximately 15 m deep during neap tides).

The parameters measured and the units expressed include water temperature (°C) and salinity (‰), using a temperature-salinometer (Yellow Springs Instruments); dissolved oxygen (mg/l) using a D.O. meter (YSI); and the nutrients ortho-phosphate-phosphorus and nitrite-nitrogen (µg atom/l) determinations with a spectrophotometer (Tokyo Photoelectric Co.) after reacting the orthophosphate by the stannous chloride method and nitrite by the diazotization method (Anon, 1971).

The managerial staff at the Kuri Bay lease site also made available oyster mortality observations recorded on a transportion trip by trip basis for the fishing seasons from 1976 through 1982 (K. Ochiai pers. comm.). Mortality records for 1976 and 1977 were not used in this report as the staff was experimenting with other culture methods and were not consistent with their methods of observing mortality. From the 1978 season onwards, however, careful observations on mortality were made on subsamples from each collection trip on a weekly basis after the arrival of the oysters at the culture lease.

For each of these trips, the staff recorded the boat used for transport, the day of receipt, the total number of oysters received at the lease, the size composition of these oysters in terms of small, medium and large oysters and the location of the raft where the oysters were suspended. After the oysters were sorted and grouped into the three sizes on the raft, usually one pole (sometimes two) of ten baskets i.e. 100 oysters, was selected at random and tagged for each size group. The baskets on these poles were raised weekly and the number of dead oysters counted. The mortality observations for each trip continued between 23 and 74 days and formed the managerial staff's means of estimating mortality for oysters on the raft until all the dead oysters were individually counted during the first cleaning operation to remove fouling organisms approximately three months after their receipt.

The above company mortality records have been placed on computer file by the authors for the years 1978 through 1982. The percentage mortality for each size subsample for each trip over this period, for means of comparison, was obtained from the number of dead oysters on the observation date nearest to 40 days after receipt of the oysters at the lease site. This time period was used to take account of the observed plateau in the pattern of mortality after approximately six weeks. The observations on subsamples from one trip which had been held less than 29 days were excluded from further analysis. The company's daily water temperature records from the Kuri Bay lease site as well as their water temperature records from the fishing grounds were also placed on computer file.

The Kuri Bay lease site settlement also acts as a weather station. Records of average monthly air temperature and monthly rainfall totals for the period January 1977 through April 1982 were obtained from the Bureau of Meteorology and placed on computer file for correlation and regression analyses with other environmental parameters.

1.4 Manipulative field experiments

Oysters for various field experiments were obtained by accompanying Pearls Pty Ltd's transport ships to the fishing grounds and setting aside the required number of pearl oysters as they were brought on board from the pearling luggers and were being sorted into the three size categories. Baskets of oysters for a given treatment were tagged either with various brightly coloured plastic strips or with a numbered plastic sheep ear-tag clipped onto the side of the basket. The individual oysters in a given basket were assigned numbers one through ten on the basis of their position within the basket from left to right.

Upon arrival at the Kuri Bay lease site, these tagged baskets were unloaded from the carrier tanks and suspended from the raft in randomized positions along with the other oysters from that collection trip. These experimental positions were tagged with coloured plastic strips to prevent disturbance by company workers. Counts were made over the following weeks on the number of dead oysters. The surviving oysters at the finish of each experiment then reverted back into the company's culture system.

1.4.1 Size, depth and cleaning experiment

The first major field experiment was designed to determine the effects on mortality of oysters of different sizes, of the different depths at which they were suspended from the raft at the lease site, and of the removal of fouling organisms. A total of 900 oysters (90 baskets) were separated into 18 treatments (5 baskets each) consisting of three sizes measured dorso-ventrally from the shell valve hinge to the growth edge (small <102 mm; medium 102 to 140 mm; and large >140 mm); three depths (2, 6 and 10 m); and whether the shell had been cleaned of fouling organisms as is the normal procedure or left uncleaned when collected.

The baskets of oysters were suspended under the northwest corner of the Kuri Bay C raft on 4 May 1981. The cumulative number of dead oysters in each treatment was recorded in the final of ten observational checks on 24 July 1981 (after 81 days suspension on the raft).

1.4.2 Contagion experiment

A contagion experiment was also set up on 4 May 1981 on the adjoining northeast corner of the above raft to see if the distribution of mortality in a large number of similarly treated oysters resulted in a clumped pattern (implying some

infectious agent being transmitted to close neighbouring oysters) or a binomial distribution pattern (implying a randomised individual mortality). A total of 900 large oysters (90 baskets) was suspended from nine consecutive poles at 5-6 m depth i.e. the normal handling procedure. Individual oyster mortality was checked on ten occasions over the period until 24 July 1981 when the experiment finished (after 81 days on the raft).

1.4.3 Density experiment

To determine the effects of oyster density within single baskets on initial mortality after collection, an experiment was conducted between 4 May and 21 June 1982 (48 days on the raft). In a randomized block design, this experiment compared the mortality of three oyster sizes (small, medium and large) in baskets containing densities of two, four, six, eight and ten oysters. The oysters within a given basket were spaced as distantly apart from one another as possible i.e. baskets with a density of only two oysters had oysters in the partitions at either end, whereas all the partitions were filled in baskets with the normal complement of ten oysters.

For each of the three size categories, a total of 192 oysters was allocated to 41 baskets in the following manner: 15 baskets with a density of only 2 oysters; 10 baskets of 4 oysters; 7 baskets of 6 oysters; 5 baskets of 8 oysters; and 4 baskets of 10 oysters. At the end of the experiment the number of dead oysters per basket was counted.

1.4.4 Long-line experiment

From preliminary analysis of the 1981 pearl oyster mortality observations on a transportation trip by trip basis furnished by the Kuri Bay staff, the mortality rate of oysters appeared greatest following transport during the winter months (June through August) compared with the rest of the year. Their records also showed that the difference in water temperature between the fishing grounds and their lease site was about six degrees Celsius during the winter whereas at the beginning (April) and end (November) of the fishing season, the difference was only one or two degrees Celsius.

To test the hypothesis that mortality would be less in oysters of all three size categories transported when the water temperature difference was minimal, oysters were placed during August 1982 on one long-line at Cape Villaret near the collection grounds (Fig. 1) and on a similar long-line at the lease site near Kuri Bay C Place as well as on a nearby raft in the normal manner. Then a third of the oysters from both long-lines were transferred to this raft at monthly intervals as the water temperature difference between sites decreased.

Both long-lines were constructed using approximately 150 m of 24 mm diameter black polyethylene monofilament rope with

flotation provided by 36 cm diameter black polyethylene floats. Fifty floats were spaced three metres apart along the long-line with four additional floats clustered together at either end to buoy-up the anchor ropes. To withstand the large tidal range and associated strong water movements, the two anchor ropes were each roughly 60 m long in order to be five times the water depth of 12 m at low tide. These anchor ropes, also of 24 mm black polyethylene, were attached to 5 m heavy chain which was in turn shackled to an 80 kg anchor.

On each long-line, 5 m lengths of 12 mm black polyethylene rope were attached under the fifty floats, as well as midway between floats, so that the baskets could be suspended at the usual 5 m depth and be spaced 1.5 m apart.

To equalize some of the differences in the amount of time spent aboard the transport ship, the baskets of oysters for the Cape Villaret long-line were tagged on the next to last day of collection and the oysters for the Kuri Bay long-line and raft were tagged on the last day of collection i.e. before departure. Then as the oysters were placed on the long-line at Cape Villaret during a stopover of a few hours on the way to Kuri Bay, all three sets of oysters had been initially aboard the transport ship approximately two days.

A total of 2100 oysters were collected on 4 and 5 July 1982. On 6 July, 900 oysters (300 of each size) were suspended from the Cape Villaret long-line; a similar 900 oysters were placed on the long-line at Kuri Bay as well as the remaining 300 oysters (100 of each size) on the raft there on 7 July 1982.

One hundred oysters of each size were transferred from each long-line to the raft on 17 August (after 42 days suspension). This movement was repeated on 14 September (after 70 days suspension); and the last third of both long-line sets of oysters were transferred on 13 October (afer 99 days suspension). The final mortality count was made at the conclusion of the experiment on 27 November 1982 (after a total of 144 days suspension).

1.4.5 Transport experiment

In order to examine the effect of time of transport, position in the transport tanks (i.e. bottom, middle or top level) and position in which the oyster is carried (i.e. vertical or horizontal) on the incidence of mortality,the following experiments were conducted on two transport vessels in May 1982. On the first day of oyster collection 10 baskets each containing 10 oysters were tagged then placed on the bottom of the tank. Ten more baskets were marked and placed on the middle level and 10 more on the top level. The procedure was repeated on the 3rd day of collection in a different tank. The same procedure was carried out on the second boat on the same neap tide and repeated on both boats for the first day of collection at later neap tides.

To compare mortality between oysters carried in a horizontal position to those carried in the conventional vertical

position 20 baskets were marked and placed vertically (oysters horizontal) on the bottom and 20 more baskets vertically on the top row of the tank on the first day of collection.

All oysters were treated in the same manner on the lease site at Kuri Bay.

The mortalities, determined in June and July 1982, were compared to the average recorded mortalities for these trips.

2. Investigation of disease

Oysters used for experimental purposes were obtained from the 80-Mile Beach and from leases in Roebuck Bay and Kuri Bay. Transport to Broome from the 80-Mile Beach and Kuri Bay was in transport tanks in carrier vessels. Oysters were packed in plastic bags, placed in cartons and flown to Perth.

Oysters used for study of the natural disease were obtained from the 80-Mile Beach and from rafts at the Kuri Bay lease. Fifty normal oysters were examined immediately after they were removed from the bottom at the 80-Mile Beach. One hundred and fifty sick oysters were examined pathologically but only 53 were examined both pathologically and bacteriologically.

In the field and in a number of experiments haemolymph was aspirated from the pericardial sac and/or surrounding coelom. After wedging the oyster open a hypodermic needle with stilette (19-21 g x 8 - 10 cm) was inserted between the valves approximately 2/3 of the distance from the posterior ear and the posterior margin. The needle was pushed through the adductor muscle above the rectum until the pericardial cavity was reached. The stilette was withdrawn and haemolymph aspirated into a 1 ml syringe.

2.1 Pathological procedures

For examination oysters were held open by placing a wedge between the valves. The top valve was removed by cutting around the pallial line and through the adductor muscle with a sterile scalpel. Samples of mantle, ctenidium, heart, digestive gland, several areas of gut, labial palps, byssus gland and adductor muscle were fixed in Davidson's fixative. Tissues were processed routinely in paraffin and sections stained with haematoxylin and eosin (H E). Selected sections were stained with Gram Twort, Ziehl Neelsen, toluidine blue, Perl's Prussian blue, Schmorl's, phloxine tartrazine, Feulgen and methyl green pyronin stains and by the periodic acid-Schiff, acid phosphatase and beta glucuronidase reactions.

Heart, digestive gland, pericardial gland and gut were prepared for electron microscopy by mincing in cold 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2. After 12-24 hours fixation tissues were rinsed in 0.1M sodium cacodylate/0.12M sodium chloride buffer and post-fixed in 1% osmium tetroxide in the above buffer. Tissues were processed, embedded in Epon and examined in a Philips 200 electron microscope.

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2.2 Bacteriological methods

Sterile swabs were used for sampling tissues for bacteriology. Tissue covering the pericardial and body cavities was opened using sterilized instruments. Haemolymph was sampled from the pericardial cavity. The surface of the digestive gland was incised with a scalpel and then swabbed as far away from the gut as possible. Swabs were then taken from the incised gut. When muscle was examined a small block of tissue was removed, seared in boiling water and incubated in broth.

Media used were nutrient agar (NA), NA + 2.5% sodium chloride, TCBS (thiosulphate-citrate-bile salts) solid media and NA + 2.5% sodium chloride broth.

Selected colonies were cultured on sheep blood agar and NA with 1%, 6%, 8%, 10% and 11% sodium chloride.

Cultures were examined for physical and biochemical characters and antibiotic sensitivity (Table 1).

In the laboratory, media were incubated at 37°C. In the field, media were incubated at ambient temperature which varied with time of day and seasonally. Ambient temperatures ranged from 25°-34°C.

Growth of one isolate (KB9/81) was examined at 18° and 37°C in NA + 2.5% NaCl broth. Bacterial numbers as colony forming units per millilitre (cfu/ml) were counted by spreading a known volume of NA broth at 6 and 24 hours incubation onto TCBS which was incubated at 37°C for 24 hours.

2.3 Virological procedures

Virus isolation was attempted from 10 normal and 26 diseased oysters. After the upper valve had been removed, haemolymph, heart and a portion of digestive gland were removed aseptically. Samples obtained in the field were frozen in liquid nitrogen until processed. Fresh tissue was processed from normal oysters.

Virus isolation was performed by Dr J. Copland, Regional Veterinary Laboratory, Department of Agriculture, Benalla, Victoria. Heart, digestive gland and haemolymph were used for inoculation of tissue cultures. Tissues were homogenized. Heart tissue was divided and half was filtered through a 0.45 micron membrane filter. The remaining half, digestive gland and haemolymph were treated with 1000 i.u. penicillin and 1000 µg streptomycin/ml. Inocula were diluted 1:5 weight/volume with MEM Earle's BSS with 2% foetal calf serum, pH 7.6. Aliquots (0.2 ml) were inoculated onto duplicate RTG-2, FHM, CAR, BF-2, Grunt fin and BB fish cell lines and incubated at 24°. Two further passages were made at 10 days duration.

2.4 Bacterial counts of water in the carrier tanks during transport.

Seawater off the 80-Mile Beach was collected by the water-sampling apparatus referred to above. Samples were obtained at high tide, twice daily for 3 days at a depth of 1-2 metres. The water was collected on the windward side of the transport vessels.

Samples of water in the transport tanks were collected in a 2 gallon bucket that was thoroughly cleaned and rinsed in fresh-water prior to use. Surface water was collected twice daily, at the same times, during the oyster collection period and during transport to the lease. The morning samples during the collection period were obtained after the boat had been anchored for 12 hours. The evening samples were collected after the last batch of oysters had been placed in the tanks.

Water samples were obtained from chlorinated water in the tanks prior to the collection period and immediately after the oysters had been removed at the lease. Another sample was obtained from the water remaining in the tank after the tanks had been emptied.

A known volume of water was filtered through a sterile 0.45 micron membrane filter held in a sterile Nalgene filter units referred to above. The membrane filter was placed on to TCBS agar and incubated at ambient temperature (28° - 32°) for 24 hrs. Bacterial colonies on the filter were counted and results expressed as bacteria per ml of water.

3. Experimental protocol for transmission and other laboratory experiments

3.1 Field transmission experiment

Attempts to transmit disease were performed using 90 oysters at Kuri bay. All oysters used had been in the same environment for at least 9 months.

Two pools of inocula were prepared from diseased oysters and one pool (control) from normal oysters. Oysters were opened as noted previously. Haemolymph was aspirated into a sterile syringe. Heart and a portion of digestive gland remote from the gut lumen were removed aseptically. Tissue and haemolymph were homogenized in Dounce homogenizers. Sterile 0.9% saline was used as diluent. The homogenate was filtered through several layers of sterile gauze to remove debris and then half centrifuged at 4000 rpm for 15 minutes and then was filtered through a 0.45 micron membrane filter. Filtrates were cultured on NA + 2.5% salt and TCBS agar.

Gauze-filtered and membrane-filtered inocula from the two pools of diseased oyster tissue and control tissue were inoculated into the mantle cavity, the adductor muscle (intramuscular) and into the haemolymph of normal oysters. Groups of 5 oysters were used for each method of inoculation. Forty five oysters were left at Kuri Bay and 45 were transported to Western Australian Marine Research Laboratories and held in flowing seawater at 27°, this being the water temperature at Kuri Bay at that time. Oysters were observed for 40 days.

3.2 Laboratory experiments

At Murdoch University oysters were held in 30 x 61 cm or 36 x 91 cm glass aquaria with a quartz gravel filtration system. Tanks were housed in environmentally controlled rooms. No food was given but previous experience had shown that oysters remain viable for several months without food.

For experimental studies, groups of 5,6 or 13 oysters were used for test and control. All oysters used in an experiment had been treated in the same manner prior to the experiment. In most cases oysters had been held for 4-8 weeks in the laboratory before being used. Preliminary studies to those listed in Table 2 are presented in Appendix 1.

3.2.1 Bacterial inocula

The following bacteria were used: *Vibrio sp.* KB 9/81, KB 7/82 and *Pseudomonas putrefaciens*. The Vibrios had been isolated from the haemolymph of sick oysters at Kuri Bay and were later identified as *V. harveyi*. *P. putrifaciens* was isolated from the haemolymph of diseased oysters during preliminary investigations in the laboratory. (see Appendix 1).

Bacteria were grown in 100 ml aliquots of nutrient broth + 2.5% NaCl at 37°. Six-hour cultures were centrifuged, washed three times in sterile saline and resuspended in 100 ml sterile saline.

Bacterial concentration was determined by a standard serial dilution technique using NA + 2.5% NaCl as the medium. Results were expressed as cfu/ml.

3.2.2 Experimental reproduction of disease

Experiments 1 - 13 were performed to establish whether isolates KB 9/81, KB 7/82 and *P. putrefaciens* were pathogenic for oysters and to determine whether pathogenicity differed in oysters held at 19° and 29°.

The protocol of Experiments 1-13 is presented in Table 2. Pathological and bacteriological procedures were performed as indicated above unless the oysters were used for Condition Index studies (below).

3.3 Bacterial invasion

The following experiment was designed to determine when the timing of invasion of oyster tissues during the period of infection with *Vibrio* sp.. Eighteen oysters used had been held at 21° without food for several weeks. Two oysters had been held at 19°. The oysters at 21° were bled and then were placed in water at 19°. Prior to infection, haemolymph was aspirated (bled) from 20 oysters at 19° and cultured on TCBS agar.

Preparation of inoculum and the programme of inoculation was the same as that used in previous infectivity experiments. The mean bacterial count of the inoculum was 7 x 10^{8} colony forming units (cfu)/ml. The protocol of the experiment is outlined in Table 3.

An 0.5 ml aliquot of haemolymph was spread uniformly over TCBS agar and incubated at 37°. Approximately 1 cm^{3} of adductor muscle was removed aseptically after the oyster was opened in the manner described previously. The muscle was scalded to remove surface contamination and then incubated in nutrient broth + 25% NaCl at 37° for 24 hours. The broth was subcultured onto TCBS agar.

Bacterial colonies isolated from haemolymph were counted and recorded as: No growth, slight (1-20 cfu's), moderate (21-100 cfu's), heavy (101-300 cfu's) or very heavy (>300 cfu's).

The results of muscle cultures were expressed qualitatively.

3.4 Investigation of bacterial toxinogenesis

This experiment was planned to determine whether toxins from *Vibrio* sp. and *P. putrefaciens* could produce mortality when injected into the body of the oyster.

Oysters were prepared by removing a notch of shell between the posterior ear and posterior margin adjacent to the pericardial cavity. Inocula were injected into the adductor muscle via this notch.

Vibrio sp. (KB 7/82) and *P. putrefaciens* were cultured in 10 ml nutrient broth + 2.5% NaCl at 37° for 6 to 18 hours (see Table 4) and inocula were prepared as above. Cell-free inocula were prepared by sonication. Cultures were subjected to two 60 second and one 30 second bursts with 30 second intervals.

The experimental design is presented in Table 4. Groups of 5 oysters received a cell or cell-free preparation. The size of inoculum in each case was 0.5 ml. Control oysters received 0.5 ml sterile nutrient broth.

3.5 Condition Index (CI)

The CI was determined on oysters in Experiments 7,8 and 11 (Table 2).

Oysters used for CI determination were removed from their shell by cutting the adductor and pallial muscle attachments on both valves and transferring the oysters intact body mass to a pre-weighed beaker for measurement of wet weight. Oysters were then homogenised and replicates weighed into smaller pre-weighed beakers to be frozen overnight. Frozen replicates of oyster homogenate were then freeze-dried, before being heat desiccated to constant weight.

The Condition Index was calculated as:

$$\frac{\text{Total dry oyster meat weight}}{\text{Total wet oyster meat weight}} \times 100, \text{ (Shaw } \underline{\text{et}} \text{ } \underline{\text{al}}\text{., 1967).}$$

4. Analytical methods

The experimental factors affecting the proportions (p_i) of oysters which were dead at a specified time period were examined using a linear logit model, log (p_i (1-p_i)). This analysis assumes a binomial error distribution which is generally considered appropriate in considering the variations between proportions (Cox 1970). The statistical package GENSTAT (Alvey et al., 1977) which uses the maximum likelihood method of Nelder and Wedderburn (1972) to estimate the linear parameters of the models, was used to analyse the data. The significance of factors and their interactions in the models in explaining the variability in the proportions of dead oysters was determined by fitting two models, one with the factor and the other without.

The difference in the likelihood ratio chi-square statistics (G^2) of the two models with the appropriate degrees of freedom (d.f.) was then used to determine the significance of the factor, providing the second model adequately fitted the raw data (Bishop et al. 1975). The ratio of the parameter estimate to its standard error can also be compared to the t distribution in examining the significance of the parameters in the model. When parameters of models which did not provide a good fit needed to be evaluated, a heterogeneity factor, G^2/d.f. (Finney 1971), was applied to the variances of the parameters.

The complementary log-log link was also considered in the analysis of the experiments. As this method gave very similar results to the logit link, only results using the logit link will be reported.

IV RESULTS

1. Field studies

1.1 Water quality parameters

Lacking published baseline data on water quality parameters for the far north Western Australian coastal region, the search for some environmental factor that might contribute to mass mortality in pearl oysters was largely a process of elimination after making initial

measurements on various parameters generally deemed important in marine biological processes. With the exception of water temperature, however, most of the water quality parameters sampled at Kuri Bay during our investigation between the period May 1981 and April 1983 failed to reveal any major anomalies from sea water sampled at the other pearling lease sites or indeed from the fishing grounds off Eighty Mile Beach. The availability of the pearl oyster's phytoplanktonic food (quantified by measuring chlorophyll a) as well as possible limiting nutrients for this food supply at Kuri Bay, for example, appeared to differ little from the range of values obtained from the other lease sites or fishing grounds (Table 5). For comparative purposes, this table also shows the range of values for these parameters from other coastal locations that have been studied in Western Australia. Noting that the upper ranges of some of these parameters may be extended in areas of high natural productivity such as the Abrolhos Islands or where nutrient input from man-made waste occurs as in Cockburn Sound and Owen Anchorage, most of the remaining differences probably reflect sampling intensity of collection rather than major differences between Kuri Bay and the other sites.

As all the culture lease sites were strictly marine in character, large fluctuations in values of water quality parameters such as salinity were not observed as might have been expected in more estuarine situations. The measured salinity at all sites only ranged between a temperature corrected specific gravity value of 1.0242 g/cm^3 (equivalent to a salinity of 32.7 parts per thousand) and a value of 1.0267 g/cm^3 (35.9%).

While the Secchi disc readings ranged between 2.0 - 11.5 m, the turbidity at Kuri Bay and the other lease sites is greatly influenced by the large tidal range in the Northwest. The tides are semidiurnal i.e. a tidal cycle with two high and two low sea levels per day, with large neap to spring variation. The average spring-tide range at Broome, for example, is 8.2 m; the maximum range is 11 m while the average neap range is only 1.8 m (Wright et al., 1982). Even at the most distant oceanic sampling site on the fishing grounds, the turbidity caused by tidal movement restricts diving effort to neap tide periods of improved visibility.

No hydrogen sulfide was detected in any of the bottom water samples taken beneath oyster rafts on the various lease sites (Judi Hansen, pers. comm.). The production of hydrogen sulfide can arise through anaerobic bacterial reduction of sulfate in sea water in anaerobic conditions when sufficient organic matter is present or through bacterial decomposition of organic matter. The strong tidal movement probably inhibited both mechanisms by preventing anaerobiosis and the build-up of oyster faecal material underneath the rafts. Hydrogen sulfide has adversely affected the physiological activities of oysters in Japan on lease sites where rafts have been positioned closely together in comparatively calm waters (Imai, 1977).

Lack of dissolved oxygen was not implicated as a cause of mortality as the minimum level of oxygen detected either in the carrier tanks full of oysters during transport or under the rafts at the depth the baskets of oysters were suspended was 5.8 parts per million or a level corresponding to 88% of air saturation. Samples included readings taken under the rafts just before sunrise when the oxygen contribution from photosynthetic activity would be expected to be at a minimum. Sessile adult bivalves generally have low respiratory requirements and edible oysters, for example, are known to survive for up to five days in water containing less than 1.0 ppm oxygen (Sparks et al., 1958).

Kuri Bay's isolated location effectively precluded environmental degradation due to urban and industrial pollution. Since 1975 Pearls Pty Ltd has further reduced man-made pollution near their lease site by even restricting the use of synthetic detergents and ensuring that any rubbish was burned and buried ashore rather than dumped into the water at the lease (H. Kan-No and H. Uemoto, pers. comm.).

A number of potentially toxic phytoplankton species have been recognised in the tropical waters off Northwest Australia belonging to the genera *Gymnodinium*, *Gyrodinium*, *Gonyaulax*, *Prorocentrum*, and *Trichodesmium* (G. Hallegraeff, pers. comm.). In keeping with the general characteristics of tropical communities, very large numbers of species are present, but usually with only small populations of each. The most striking phenomenon is annual surface blooms of *Trichodesmium* sp. along the coast especially during October and November when the wind is slight. Unlike the often heavy mortality of pearl oysters associated with red-tides in Japan, the decay of *Trichodesmium* after blooms has not been noted to cause unusual mortalities of pearl oysters on leases in Australia or where it has been observed on coastal leases in India (Chellum and Alagarswami, 1978).

Given the non-estuarine locations of the culture leases and the lack of apparent anomalies in the above water quality parameters, attention was focused on water temperature. Records kept by the Japanese staff at Kuri Bay showed a water temperature difference during the winter months between the collection grounds and their lease site of almost 7°C. This large temperature difference that the oysters are subjected to during transport approaches the maximum annual variation of not more than 10°C at any place in the open ocean (Nicol, 1960). A temperature difference was confirmed by our sampling in June 1982 when the surface water temperature at the fishing grounds was 21.5°C; and upon arrival at Kuri Bay a few days later, a surface temperature of 25.8°C was recorded.

1.2 Industry records

As far as is known, no pearl culture company other than Pearls Pty Ltd has kept systematic records of any environment water quality parameter. Fortunately, the Japanese biological staff at Kuri Bay h been instructed to keep records of monthly water samples taken at the same raft site stations since 1978 as part of their

attempt to improve the seasonal timing of their nucleus implantation operations and as background data for their attempt to propagate pearl oyster spat. These records were made available for perusal for this study (M. Yasui, pers. comm.). Some of the data, such as their recording for pH and dissolved oxygen, appeared to fall well within the normal range of sea water criteria (Poxton and Allouse, 1982) and were not analysed further.

Computor time series plottings were made of their monthly records of the nutrients orthophosphate and nitrite and the major water quality parameters of temperature and salinity around the six raft sites for the period March 1977 through April 1982. An example of this plotting for the raft site which was used for most of the experimental field work appears in Figure 2.

The corresponding average air temperature and monthly rainfall totals at Kuri Bay obtained from the Department of Meteorology for the period January 1977 through April 1982 are plotted in Figures 3A and 3B respectively.

A similar plot of PPL records for percentage pearl oyster mortality averaged over three size classes on a transportation trip by trip basis for the years 1978 through 1982 is given in Figure 4.

In a multiple regression analysis of parameters related to the above oyster mortality records for this five year period, the percentage of the variance accounted for by oyster size and year to year differences was 35.0%. When the environmental parameter, water temperature, was added to the equation, 55.4% of the variance was explained. When the rest of the above environmental parameters were added, the percentage increased only to 56.8%. Thus water temperature was considered to be the most important environmental parameter related to mortality.

Using the water temperature records collected by the PPL staff during the extended 1981 fishing season, the differences in surface water temperatures between the collection grounds and their lease site in the middle of the year are apparent in Figure 5.

The increase in the percentage mortality of collected pearl oysters transported during the middle of the 1981 season is shown in Figure 6.

Linear regressions of the surface water temperature records between the collection grounds and their lease site for the years 1979 through 1981 show strong correlations between seasonal temperature changes at either site (Figure 7). For this reason, the more extensive water temperature records from the Kuri Bay lease site were used to examine the importance of temperature component of oyster mortality.

The percentage mortality for oysters of each of the three size categories during the 1981 season is inversely related to water temperature (Figure 8). Analysis of covariance of the linear

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regressions of percentage mortality for each size category demonstrated no significant differences between slopes ($F_{2,107}$= 1.12, n.s.), but a highly significant difference between elevations ($F_{2,109}$= 39.44, P<0.001). Similar results were obtained for linear regressions of trip by trip mortality records for the year 1980 (for slope $F_{2,93}$ = 1.18, n.s.; for elevation, $F_{2,95}$=8.44, P<0.001) and for the year 1982 (for slope, $F_{2,60}$=0.58,n.s.; for elevation, $F_{2,62}$=6.27, P<0.01). Thus, while all three oyster size categories exhibit mortality that is inversely related to water temperature at the time of transport, a higher percentage of the smaller sized oysters die over the entire season than do larger sized oysters for unknown reasons.

The mortality data for the year 1981 (Figure 6) was further examined for seasonal effects by comparing the first half of the fishing period with the second half using analysis of covariance. After the effects of oyster size and water temperature were taken into account, no significant difference in mortality between oysters transported at the beginning of the fishing season compared with those transported during the latter half was detected ($F_{2,105}$=1.55,n.s.). Significant seasonal effects might be expected if the mortality resulted from some other stressful annual physiological process such as spawning as was implicated in mass mortalities of edible oysters in Japan (Sindermann, 1979).

1.3 Manipulative field experiments

1.3.1 Size, depth and cleaning experiment

In the linear logit analysis of oyster mortality in the experiment of different sized oysters, cleaned or left uncleaned of fouling organisms and suspended at different depths, no significant difference was found in the cleaning factor. Oyster size, however, was found to be very significant (P<0.001) with the percentage mortality decreasing inversely with oyster size (small 72.7%; medium 63.6%; and large 39.0%).

Whilst the depth categories of 2 m and 5 m were not found to be significantly different, the depth category of 10 m was found to be significantly different (P<0.05) with less mortality found at the 10 m depth compared with 2 and 5 m. This result, however, was not consistent over all replicates of the experiment as there was also a significant interaction between depth and replicates which is probably due to the positioning of the replicates on the raft.

1.3.2 Contagion experiment

The results of the contagion experiment indicated that the disease was not contagious. The mean number of dead per basket was 4.92 (with sample variance of 3.20) from 90 baskets of large oysters. The distributon of the number of dead per basket was fitted to a binomial distribution in order to detect evidence of non-randomness and resulted in a good fit (P>0.05). A second test for non- randomness, the binomial index of dispersion (Seber, 1973), which is based on comparing the observed variance estimate with an estimate of the expected variance under a binomial model, was also used. This

test resulted in the difference between the two variances which was just significant at the 95 percent level. However, an analysis of the rows and columns where the baskets were placed on the raft indicated that the two rows along the outside edge of the experimental group next to other non-experimental oysters had a higher mortality than the other oysters in the experiment, and when these were removed from the binomial index of dispersion test, no significant difference was detected between the two variances indicating that in general there was no evidence of non-randomness i.e. no contagion in this experiment.

1.3.3 Density experiment

In the experiment of oyster density within baskets, the mortality in baskets of 10 oysters, when compared to other basket densities (2 to 8 oysters), was found to be significantly greater ($P<0.01$) with a G^2 of 7.4 with 1 degree of freedom. As an example, the expected percentage mortality of medium sized oysters in densities of 10 oysters per basket was 48%, whereas in baskets of lesser densities the expected mortality was only 34%.

1.3.4 Long-line experiment

In the long-line experiment, after 42 days suspension, there were significant differences in percentage survival (100 minus percentage mortality) of the three sizes of oysters ($P<0.001$) and at the three sites ($P<0.001$). The Cape Villaret long-line site recorded no mortality for any size category while the Kuri Bay long-line had less mortality than the Kuri Bay raft (Table 6).

At the end of this experiment (144 days suspension), the last third of the Cape Villaret long-line oysters, transferred to the Kuri Bay raft when water temperature differences between sites were minimal, recorded 100% survival for all size categories while there was 60-80% survival recorded for the Kuri Bay long-line oysters transferred to the raft in Kuri Bay during the same period (Table 7). There was a general increase in survival the longer oysters were left on the long-lines. There was, however, significant interaction ($P<0.001$) between the site of long-line and day of transfer, mainly due to the fact that for the samples moved at 42 days, the Kuri Bay long-line oysters showed a higher survival than those from the Cape Villaret long-line.

1.3.5 Effects of transport time and position during carriage

The results are presented in Table 8. On the one trip in which time within the tanks was evaluated the mortality was significantly lower ($P<0.01$) in oysters loaded on the third day of collection than those loaded on the first day of collection.

The results relating to position are conflicting. There was no significant difference in the mortality rate between oysters carried on the bottom, middle or top of the tanks in two of the four trials. In trial 3 there was a significantly greater mortality in oysters carried on the bottom and in trial 4 a significantly greater mortality in oysters carried on top of the tank.

There was no significant difference in mortality in oysters carried in the horizontal position when compared to those carried vertically.

2. Investigation of disease

There was no evidence of excessive mortality in oysters in their natural environment. Morbidity and/or mortality occurred after arrival at the lease sites. The cumulative mortality at times after arrival is presented in Fig. 9. In general there was little or no mortality within the first 7 days and then a gradual or rapid rise over the next 30-40 days. The incidence of mortality rate varied considerably for oysters collected within and between neap tides and was greatest during the winter months (Fig. 6).

The rate of mortality varied inversely with oyster size (Fig. 8). Small oysters (< 102 mm) sickened and/or died at a greater rate than medium (102-140 mm) and large oysters (> 145 mm).

2.1 Pathology

Diseased oysters were recognized by gaping, withdrawal of the mantle and dirty, stained nacre between the withdrawn mantle and the edge of the shell. The degree of these changes was variable. In many cases gaping oysters closed rapidly upon stimulation but soon reopened. Others closed slowly and when closed the valves were not completely apposed. Those oysters that did not close but still attempted closure and had obvious ctenidial activity and heart beat on opening were classed as moribund. Dead oysters gaped fully and putrefied rapidly but ctenidial activity was occasionally seen in these oysters.

Visible lesions were not apparent on gross examination. However, histological examination revealed lesions in 74% of oysters examined. Variable sized focal accumulations of phagocytic haemocytes were present in many tissues particularly the connective tissues of the digestive gland (Fig. 10) and mantle. Generalized or focal accumulation of phagocytic haemocytes were commonly present in the auricle (Fig. 11) and ventricle. A common finding was erosion of the epithelium of the outer fold of the mantle and the marginal zone (Fig. 12) and infiltration of the subepithelial tissue with phagocytic cells. Gram negative bacteria were occasionally seen within phagocytes in all tissues.

Atrophy of glandular epithelium of digestive gland tubules and dilation of tubules was common.

Ultrastructural examination revealed no more than light microscopic findings.

Other histological findings that were seen in normal and diseased oysters but were not related to disease are referred to in Appendix 2.

2.2 Bacteriology

Haemolymph from normal oysters sampled at the 80-Mile Beach was bacteriologically sterile. From 53 sick oysters bacteria were cultured on primary isolation from the haemolymph of 40 (75%). Of these isolates 90% were identified as Vibrio sp. The number of bacteria isolated varied from a light to heavy growth (exact plate counts were not done in the field) and pure cultures were not obtained on all occasions.

Vibrio sp. were isolated in large numbers from the gut of normal and diseased oysters.

The most frequently isolated bacteria produced a smooth, convex, circular cream colony 1-2 mm diameter on NA + 2.5% NaCl at 24 hours incubation at 37°. On TCBS agar the colonies were smooth, convex, circular and yellow. Five isolates from diseased oysters in 1981 were examined in detail (Table 1). All isolates were identical and were designated KB 9/81. A similar colonial type isolated from diseased oysters in 1982 (KB 7/82) was also examined in detail (Table 1). These isolates were classed as marine Vibrio sp. (V. Bamford - State Health Laboratory Sevices, Department of Public Health, WA) and specifically as *V. harveyi* by Dr R. Sakazaki, National Institute of Health, Tokyo, Japan.*

Isolate KB9/81 grew rapidly at both 18° and 37°C although the rate of growth was slower at 18°C. At 18° the number of bacteria were 5.0×10^{8} and 5.3×10^{7} cfu/ml at 6 and 24 hours respectively and at 37° were 1.3×10^{8} and 6.9×10^{7} cfu/ml at 6 and 24 hours respectively.

Two other colonial forms were also occasionally isolated on TCBS from diseased oysters. One was a circular, raised crateriform colony that was otherwise identical to KB 9/81 and KB 7/82. The other produced a large deep yellow mucoid slowly spreading colony and was identified as *V. alginolyticus* (Dr R. Sakazaki - personal communication).

Forty-nine percent of oysters had both microscopic lesions and bacteria isolated from haemolymph. Twenty-five percent had lesions and no bacteria isolated, 23% had bacterial isolates and no lesions, and 4% of diseased oysters had neither lesions nor bacteria isolated.

2.3 Virology

Viruses were not isolated from any tissue from normal and diseased oysters.

Ultrastructural examination of tissues did not reveal any other possible aetiological agents.

*In the text, *Vibrio* sp. and *Pseudomonas* sp. denotes isolates typed to genera only.

2.4 Bacterial counts on water in carrier tanks

The numbers of Vibrio sp. in the water of the carrier tanks and the ratio of Vibrio sp. in the tank water to that in seawater at the 80 Mile Beach are presented in Table 9. The number of Vibrio sp. in the surface water of the carrier tanks rose during transport but the number varied between and within days. The number of bacteria was greatest in the water immediately after the carrying baskets had been removed and was even greater in the sludge remaining at the bottom of the tanks after they had been pumped out.

3. Experimental disease

The term "morbid" is used to denote those oysters which became abnormal during the experiments. Morbid is equivalent to "sick". The criteria used to evaluate whether an oyster was morbid were as follows: (a) slight gape, no mantle retraction: (b) mantle retraction, no gape; (c) gape but responds to photostimuli; (d) gape but responds to vibration; (e) gape but responds to direct stimuli; (f) gape and mild mantle retraction; (g) gape and severe mantle retraction; (h) edge of mantle folded; (i) no reaction to stimuli; (j) reacts only when out of water; (k) slime masses in mantle cavity; (l) wide gape.

3.1 Field transmission experiments

Morbidity/mortality did not occur in any group of inoculated oysters held either in Kuri Bay or transferred from Kuri Bay to Perth.

3.2 Laboratory experiments

The results of laboratory experiments are summarised in Table 10. Disease was not produced in all experiments. In experiments 1,2,3,9 and 10 there was a greater level of morbidity/-mortality in infected than in control oysters. This occurred at both 19° and 29°. In experiments 4,5,6 and 8 there was little difference in the level of morbidity/mortality between infected and control animals. In experiments 7 and 11 there was little difference between infected and non-infected oysters at 19° or 29° but overall, the morbidity/mortality of both infected and control animals was greater at 19° than 29°.

The morbidity/mortality data were combined for all the above experiments (Table 11). When evaluated using linear logit analysis, the morbidity exhibited by control oysters was significantly less than oysters infected with *V. harveyi* ($P<0.001$). In the same analysis, significantly less morbidity was exhibited by oysters kept at 29° compared with those held at 19° ($P<0.001$). The proportion of mortality was significantly less in control oysters compared to infected oysters ($P<0.05$), and in oysters kept at 29°C compared with those held at 19° ($P<0.001$).

P. putrefaciens produced 80% and 100% morbidity/mortality at 19° and 29° respectively.

Vibrio sp. were recovered from haemolymph in oysters that were morbid in experiments where cultures of *V. harveyi* were used as inocula. In Experiments 12 and 13 where the inoculum was *P. putrefaciens*, both *Pseudomonas* sp. and *Vibrio* sp. were isolated from haemolymph.

Vibrio sp. were commonly isolated from the haemolymph of control oysters that died when kept at 19°C.

Histological lesions were the same as those described in the natural disease.

3.3 Bacterial invasion

The results of this experiment are presented in Table 12. It is evident that at 19° low numbers of bacteria can be isolated from the haemolymph of some oysters which are otherwise normal. Following administration of Vibrio cultures to the mantle cavity, the number of oysters with bacteria in haemolymph increased over the experimental period. A greater percentage of treated oysters than controls had large numbers of bacteria in the haemolymph. Bacteria were recovered from muscle in a greater percentage of treated than control oysters. All treated oysters died compared to one death in the control group. The results suggest that following administration of *Vibrio* sp. the bacteria invade tissues of the oyster.

3.4 Investigation of bacterial toxinogenesis

The results of this experiment are presented in Table 13. Intramuscular inoculation of washed *V. harveyi* (KB 7/82) bacteria and 6 and 18 hour cell-free cultures produced 100% mortality in oysters from 2-8 days after the start of the inoculation programme. Inoculation of washed *P. putrefaciens* and a 6 hour cell-free culture produced lower morbidity (80%) and mortality (40% and 0%) respectively than the *Vibrio* sp.

The lesions seen in oysters from all groups were the same as those seen in the natural disease except that a cellular response was often present within vessels and connective tissues of the adductor muscle.

3.5 Condition Index (CI)

Determination of CI of oysters held at 19° and 29° experiments 7 an 8 (Table 2) demonstrated a trend towards higher indices in oysters held at 19°. In experiment 8, treated oysters at 19° had the highest CI overall (Table 14). In experiment 7, both the CI of controls and treated at 19° were greater than the CI's of oysters at 29°C, with control oysters at 19° having the highest CI overall.

Experiments 7 and 8 were replicated in experiment 11 (Table 2), however the sample size was increased from 5 to 13 oysters per treatment. Results of experiment 11 were comparable with the earlier experiments, with oysters held at 19° having significantly higher CI's than those at 29° (pooled control and treated values at each temperature; $P<0.01$). No significant difference was observed between oysters within each temperature group, however there was a significant difference between control oysters at each temperature (19° controls versus 29° controls; $P<0.01$) and between treated oysters at each temperature (19° treated versus 29° treated; $P<0.05$).

V DISCUSSION

Disease may be defined as any change from normal structure or function of a living organism and as such is manifest in many ways and has multiple causalities (aetiologies). Disease may be due to infectious agents such as bacteria, viruses, protozoa and fungi. Non-infectious aetiologies include chemical toxins, physical agents, and hormonal, metabolic or genetic abnormalities. Most diseases have a multifactorial causality. Often a major factor is responsible but in most instances disease will not be evident unless other predisposing factors, act on the organism.

Death, or mortality, is the most overt manifestation of disease. All populations have a basic or "normal" level of mortality. Mass mortalities are regarded as an exaggerated form of natural mortality (Sinderman, 1979) and are often rapid in onset and affect a large proportion of the population over a wide geographic area. However, localized mass mortalities in restricted geographic areas do occur. In most cases mass mortalities in oysters are probably due to interactions of environmental and biological factors, the latter in some instances being infectious. However, only a few mass mortalities have been definitely associated with specific pathogens, predators or environmental factors (Sprague, 1971; Sinderman 1979).

Mortality in pearl oysters in the northwest of Western Australia could be classified as "localized mass mortalities". It was readily apparent to the industry that there was little mortality of oysters in their natural environment while mortalities greater than that regarded as "normal" occurred after oysters had been transferred to lease sites. The level of mortality considered to be "normal" by operators within the industry varied from 1 to 20% but the causes, other than predation in localized areas, were unknown.

Investigation of the mortality problem was carried out primarily with one operator within the industry. At the time of the investigation this operator was still experiencing heavy mortalities whereas the problem on other lease sites had apparently declined. Oysters were transported by ship to the lease site involved in tanks with flow through circulation. The time spent within tanks varied from 1.5 - 4.5 days depending on boat schedules and the length of neap tides.

Examination of industry records and field experiments confirmed that oyster mortality occurred after transportation. There was little or no mortality in the first week after transportation but mortality rose between the second and sixth weeks and then tended to plateau.

The highest rates of mortality occurred in the winter months. As water temperature was shown to be the most important environmental parameter related to mortality it is most likely that the high mortality in winter is associated with low water temperatures. Mortality would appear to be unrelated to other environmental parameters examined.

Analysis of industry records and the results of field experiments confirmed that mortality rate is inversely related to oyster size but the reasons for this are unknown.

The pathological studies indicated that the majority of sick oysters had an infectious disease as focal and diffuse accumulation of phagocytic haemocytes were common in various tissues in many oysters. The changes were present in oysters irrespective of whether they exhibited mild or severe signs of illness.

The bacteria recovered from the haemolymph of 75% of affected oysters were identified culturally and biochemically as marine *Vibrio* sp. Two of these isolates designated KB 9/81 and KB 7/82 have been identified as *Vibrio harveyi* (R. Sakazaki - personal communication). *V. harveyi* is a bioluminescent bacterium that occurs naturally in the marine water column, sediment and on the surface and in the gut of marine animals (O'Brien and Sizemore, 1979; Ruby and Morin, 1979). This organism has not previously been associated with disease, but disease has been produced experimentally in pearl oysters by us with this organism.

Because of the pathological, microbiological and experimental findings and as no other causal agents were found, it is considered that infection with marine *Vibrio* sp. contributes to the mortality at the lease site.

Prior to discussion on vibriosis it is pertinent at this point to comment on a suggested "protistan parasite" incriminated by Wolf and Sprague (1978) as a cause of mortality in *P. maxima*.

In 1978 Wolf and Sprague reported on the presence of an "unidentified protistan parasite" in the digestive gland of the pearl oyster. Recently, Nasr (1982) reported similar structures in the digestive gland of *Pinctada margaritifera* in the Red Sea. In both papers it was inferred that these structures were related to disease states in the oyster.

The appearance of these structures is described in Appendix 2. The light microscopic appearance, enyzme activity and ultrastructural appearance indicate that the structures are residual bodies (Owen, 1970 and 1972) which are either storage or excretory products within lysosomes. These structures are normal constituents of digestive cells and are eventually released from the cell.

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Wolf and Sprague (1978) stated that their "protistan parasite" was not present in normal oysters and that the bodies were associated with "badly disintegrated" epithelium. Although we have seen these bodies in all oysters they are often more apparent in oysters with atrophy of digestive gland and in digestive glands that are autolytic. In our experience many diseased oysters have atrophy of the digestive gland cells presumably from reduced food intake, and autolysis is common in tissues from moribund or recently dead oysters. It is possible that the "badly disintegrated" tissue described by Wolf and Sprague (1978) was in fact autolysed and not degenerate or necrotic.

We believe that the structures referred to as "protistan parasites" are not pathogens but normal constituents of the digestive cells of *P. maxima*.

Vibriosis of marine fish (Anderson and Conroy, 1970) and shellfish larvae (Tubiash et al., 1970; Brown, 1973; Brown and Losee, 1978; DiSalvo et al., 1978; Elston and Leibovitz, 1980; Jeffries, 1982) is a well recognized disease. In fish the disease is manifested by haemorrhagic septicaemia and/or an ulcerative skin disease. The organism responsible in the majority of epizootics is *V. anguillarum*. In shellfish vibriosis was first described as "bacillary necrosis" (Tubiash et al., 1965) and it occurs in naturally growing and hatchery reared larvae and juvenile cultured oysters (Elston et al., 1982) The organisms responsible for this disease tend to fall into three groups; those resembling *V. anguillarum*, those resembling *V. alginolyticus*, and a third group of largely unnamed Vibrio species.

Vibriosis of adult oysters is a less well described disease. Tubiash (1974) reported that strains of *V. anguillarum* were associated with increased rates of mortality of mature *Crassostrea virginica* but he concluded that *Vibrio* sp. were of marginal significance as primary pathogens in adult oysters.

Both *V. anguillarum* and *V. alginolyticus* have been reported to be pathogenic for mature *C. gigas* (Pacific oyster) but mortality was directly related to water temperature, mortalities being greater when oysters were stressed at high (20°C) temperate water temperatures (Grischkowsky and Liston, 1974).

The relationship between stress and bacterial disease of marine animals is well recognized (Wedermeyer et al., 1976). Stressors such as temperature, crowding, competition for nutrients, exhaustion and spawning all predispose to infectious disease. Temperature is regarded as the most important stressor affecting the balance between the organism and its environment (Roberts, 1978). Temperature has effects on both the host and on infectious agents (Kinne, 1980). Each species of marine animal has its normal temperature range and at the extremes of the range disease is more likely to occur (Roberts, 1978), the susceptibility to disease being related in part to the animals' defence mechanisms. In oysters low temperatures depress phagocytic haemocyte numbers and activity

and therefore depress cellular defence mechanisms (Feng, 1965; Tripp, 1970). The activity of cellular defence mechanisms in oysters increases with increasing temperatures (Feng, 1965) but as the upper extremes of the temperature range are reached it is likely that the efficiency of cellular defence decreases as cellular metabolic processes fail. Fish are more subject to invasion by pathogenic micro-organisms at high temperatures (Roberts, 1978) and Lipovsky and Chew (1972) demonstrated that the Pacific oyster (*C. gigas*) suffered high mortalities associated with bacterial invasion when held at high (21°C) water temperature. This may be related to the above but is probably also related to increased rate of multiplication of bacteria in the environment producing a higher "dose rate", and a greater rate of multiplication within the animal. At low temperatures although cellular defence mechanisms may be depressed, proliferation of pathogenic bacteria would also be depressed.

Vibriosis occurs most commonly in fish (Richards and Roberts, 1978), larval oysters (Elston et al., 1982) and mature oysters (Lipovsky and Chew, 1972, Grischkowsky and Liston, 1974) in cold-water environments when the temperature of the water increases. In tropical species of molluscs such as *P. maxima* the situation may be reversed. The normal temperature range for this species within this fishery is 18° to 32°. Although it remains unproven, it is likely that at the lowest temperatures cellular defence mechanisms in *P. maxima* are depressed and the oysters are "stressed". At these temperatures however, marine *Vibrio* sp. proliferate well.

Experimental Vibriosis was produced in oysters held at 19° and 29° therefore oysters were susceptible to infection over much of their normal temperature range. However it is apparent that this species is more susceptible to disease at lower temperatures because the overall rate of morbidity and mortality was greater at 19° than 29°. Also, deaths occurred in control oysters at 19° but never at 29°.

A measure of the "condition" of oysters was attempted to determine whether oysters were more subject to stress, and hence disease, at low temperatures. Stress has been defined as a measurable alteration of a physiological steady-state which is induced by an environmental change and which renders the individual more vulnerable to further environmental change (Bayne, 1975). Condition index has been used for determination of general body condition in edible oysters (Lawrence and Scott, 1982) and it is used as a measure of stress (Bayne, 1975), although it is a "rough measure" when compared to those methods dependent upon physiological or biochemical indices (Bayne 1975, Ivanovic 1980). The condition index refers to the extent to which the oyster fills its shell cavity and is traditionally measured by calculating the ratio of dry meat weight or wet meat volume to the internal cavity volume of the shell (Lawrence and Scott, 1982). The latter measurements are cumbersome, particularly with individuals as large as pearl oysters.

We have used a method of relating dry weight to wet weight to measure condition index (Shaw et al.,1967). Condition index measures the water content of tissues and is a reflection of osmo-regulatory efficiency. The results of experiments performed to calculate the condition index showed that oysters at 19° were in "poorer condition" than those at 29°. They were therefore possibly more susceptible to environmental stress and disease at lower temperatures.

The major source of infection of *Vibrio* sp.is the water column. Outbreaks of vibriosis occur as the bacterial load of the water column increases, usually in conjunction with increasing water temperature coincident with increased plankton counts (Kaneko and Colwell, 1973). The major source of bacteria for pearl oysters in the system under investigation is believed to be the water of the transport tanks on carrier vessels. It has been demonstrated that the numbers of Vibrio in the surface water of the tanks rise during transportation. In the loaded tanks only the surface waters could be sampled. The water enters the top of the tanks after entering the vessel through a venturi system. Therefore, there is more water flow as the vessel moves and this accounts for the variation in bacterial numbers during transportation. The results are not indicative of the level of bacteria deep in the tanks as the surface water is the most recently added water. The bacterial numbers remaining in the tank after unloading are probably a more accurate reflection of the bacterial numbers in water surrounding oysters deep in the tanks and it is apparent from these values that there has been massive increase in bacterial numbers during transport.

The reservoir for bacteria is most likely to be the surface and gut of the oysters. Fish faeces have been shown to be a source of infection and a medium for bacterial proliferation (Ruby and Morin,1979), hence the accumulation of excreta and other organic material in the tanks would provide a favourable environment for bacterial growth.

The other major source of infection is likely to be the water surrounding oysters on rafts at the lease sites. Preliminary (unpublished) observations indicate that the level of bacteria around oysters on rafts is much greater than that of seawater away from the rafts. Placement of oysters in this contaminated environment after they have been subjected to a high bacterial load during transport is likely to potentiate the infection. There is some evidence to support this concept in that mortality was shown, in field experiments, to be related to position of oysters on the raft and whether oysters were placed on rafts or on a long-line. Mortality was lowest in oysters placed on the outside of the raft and it was lower on oysters placed on long-lines compared with those placed on rafts. Water circulation around oysters on the periphery of rafts and on long-lines would be greater than in the centre of rafts thereby diluting bacterial numbers in these sites.

Detailed studies of the pathogenesis of Vibriosis in *P. maxima* have not been attempted but experiments designed to determine whether disease can occur following invasion of *Vibrio* sp. and

whether disease can be produced by cell-free culture products i.e. bacterial endotoxins or exotoxins of *Vibrio* sp. have been performed. The results of experiments in Table 12 indicate that *Vibrio* sp. do invade oyster tissues e.g. adductor muscle (Table 12), early in the course of the disease. The results also indicate that *Vibrio* sp. invade tissues of oysters at 19° whether or not they are exposed to high concentrations of the bacteria. The numbers of bacteria however were in general lower in tissues of control oysters than in oysters subjected to high concentrations of bacteria. Only one control oyster died whereas all oysters in the test group were moribund on day 16 of the experiment.

The site of invasion by bacteria was not examined in this study. The most likely sites are the mantle tissues, ctenidium or, following ingestion, the gut wall. Erosion of the epithelium of the shell fold of the mantle was commonly seen in the natural disease, however it is not known whether this lesion was due to penetration of bacteria from outside or from localisation of bacteria after circulating in haemolymph. Penetration of mantle tissues is however possible. Elston and Leibovitz (1980) have demonstrated three modes of infection in larval oysters which is dependent on the age/size of the larvae and on the bacterial isolate used. In their type 1 pathogenesis which occurs in all larval stages and juvenile oysters (Elston et al., 1982), bacteria attach to the periostracum of the shell and grow along mantle tissues and invade the body from the mantle.

Invasion of the epithelium of the gut is another likely possibility. Elston and Leibovitz (1980) present evidence that invasion of tissues of the gut occurs in late veliger larvae and Tubiash et al., (1965) suggested the same in their original work on bacillary necrosis of oyster larvae.

Larval disease may also be primarily produced by bacterial toxins without invasion (Brown and Losee, 1978; Elston and Leibovitz, 1980). It has been demonstrated here that cell-free extracts of Vibrio cultures will kill *P. maxima* when inoculated intramuscularly and it is probable that these toxins, whether they be endotoxins or exotoxins, act in the pathogenesis of the disease. Whether they can produce sickness or death by being absorbed from the external surfaces in the absence of invasion, as they presumably are in larvae, is not known.

The ability of *Pseudomonas putrefaciens* to produce disease in *P. maxima* was also examined. This organism was commonly isolated from the haemolymph of oysters that died in the laboratory during preliminary observations before *Vibrio* sp. were incriminated in the natural disease. Members of the genus Pseudomonas are ubiquitous in soil and water environments and exist as commensals and saprophytes (Buchanan and Gibbons, 1975) although some species are pathogenic for animals. *P. fluorescens* is a common cause of septicaemia in fish and is usually seen when fish are stressed particularly by crowding and high water temperature (Richards and Roberts, 1978). *P. enalia* has been reported to produce

disease in mature *C. gigas* experimentally (Colwell and Sparks, 1967) and it was suggested that this species is a likely secondary bacterial invader in oysters already weakened by some other disease or environmental stress. Brown (1973) showed that members of the genus Pseudomonas produced disease in *C. virginica* larvae under the same circumstances that *Vibrio* sp. produced disease.

P. putrefaciens has not previously been implicated as a cause of molluscan disease. This organism produces disease in mature *P. maxima* experimentally that is indistinguishable from that due to *Vibrio* sp. Whether natural infection with this species or other species of Pseudomonads occurs is not known; but as this genus is commonly isolated from marine substrates, infection must be considered a possibility.

VI SUMMARY AND CONCLUSIONS

(i) During the three year period (July 1980-June 1983) of this investigation, the majority of pearl oyster deaths in the culture industry occurred after transportation of oysters by the largest company from the fishing grounds to their lease site at Kuri Bay. Incidental outbreaks of oyster mortality on other lease sites were observed in less detail and were not necessarily associated with the factors described below.

(ii) Mortality in the field was related to transport. Oysters that were subjected to the same procedures following removal from the natural beds but were then not transported suffered no mortality.

(iii) Examination of industry records showed that most mortality occurred within seven weeks upon arrival at the lease site. The incidence of mortality varied within and between neap tide fishing periods but the greatest incidence occurred in the winter.

(iv) A multiple regression analysis of water quality parameters with industry records has shown that water temperature is the environmental factor most closely associated with mortality, the greatest incidence occurring when water temperatures are at a minimum.

(v) A number of field experiments were undertaken to study the effects of various treatments on pearl oyster mortality. The results were as follows:

(a) The percentage mortality was greatest in the smaller sized oysters but the reason for this is not known. Cleaning the oysters of fouling organisms did not result in a significantly different mortality rate from oysters left uncleaned. The examination of the effect of various depths at which oysters are maintained under rafts after transport was inconclusive.

(b) Mortality rates were higher in baskets with a full complement of ten oysters compared with baskets of lesser densities and higher in baskets suspended below rafts compared with baskets below long-lines.

(c) Mortality among oysters in baskets appeared to be random indicating that the disease was not contagious (see below).

(d) For samples of oysters transferred from a long-line near the fishing grounds to Kuri Bay, the highest survival rate was recorded when the water temperature difference between these sites was minimal i.e. non-winter portion of year.

(e) Increased transport time during carriage appeared to increase oyster mortality, but the results relating to position during carriage were inconclusive.

(vi) Pathological procedures were used to demonstrate that many sick oysters were suffering from an infectious disease. Although infectious i.e. caused by a micro-organism, the disease has been shown not to be contagious i.e. not transmitted directly from oyster to oyster.

Marine Vibrio bacteria were isolated from the haemolymph of sick oysters and one of the many isolates recovered has been identified as *Vibrio harveyi*. This organism has been demonstrated to cause disease experimentally and the pattern of experimental disease is similar to the natural disease. Because of the pathological and experimental findings and as no other causal agents were found, it was considered that infection with marine *Vibrio* sp. contributed to the oyster mortality at the lease site.

Structures referred to as pathogenic "unidentified protistan parasites" in earlier pearl oyster mortality studies were seen to be normal constituents of the digestive cells of *P. maxima*.

(vii) Experimental evidence suggests that oysters held at low temperture (19°C) are more stressed and therefore probably more susceptible to infectious disease than oysters held at higher temperatures. The minimum water temperature off Eighty Mile Beach is 18°C. This occurs during July and this coincides with the greatest incidence in mortality. Susceptibility to disease at low temperatures is probably related to depressed defence mechanisms of the oyster. At these temperatures, however, marine Vibrios can multiply rapidly.

(viii) It has been demonstrated that there is a rapid rise in the numbers of marine Vibrios in the water of carrier tanks during transport despite a flow-through circulation of water. The major source of Vibrios is likely to the gut of oysters and the major substrate for bacteria in the water is most probably faeces and other organic matter. It is therefore postulated that oysters, weakened by exposure to low annual ambient temperatures, come into contact with high concentrations of bacteria and that bacterial invasion occurs in many oysters during transportation. This is probably potentiated when oysters are concentrated below rafts at the lease site.

(ix) In summary it appears that the major predisposing factors associated with high mortalities in Pearls Pty Ltd's operation at Kuri Bay are cold water temperatures, concentration of oysters during transportation and inadequate water circulation in carrier tanks. A major consequence of this is infection of oysters with marine Vibrios. It must not be concluded however, that these are the only infectious agents involved or that other factors, as yet unidentified, are not involved.

(x) It is recommended to the industry that transportation of oysters be minimized during the winter months, that the number of oysters carried per trip be kept to the lowest that is economically feasible and that tank hygiene and water circulation be optimized.

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Appendix 1

Preliminary Investigations

1. Prior to incrimination of *Vibrio* sp. playing a role in oyster mortality, several transmission experiments using oyster tissues as inocula were performed.

   Oysters used were held in glass aquaria (60 x 30 or 90 x 35 cm) with a quartz gravel filtration system. Water temperature was 19° - 22°C. Oysters had not been fed for up to 6 weeks but were clinically normal. Three oysters were held in each aquarium.

   Inocula for transmission was prepared from a diseased oyster (test) and a normal oyster (control). Haemolymph, heart, digestive gland and gut, and ctenidium were homogenized and filtered through gauze. The filtrate was divided into 3 aliquots. Each aliquot was pipetted into the mantle cavity of an oyster.

   The 3 test oysters died from 10-24 days post inoculation. *Pseudomonas putrefaciens* was isolated from haemolymph on sheep blood agar (TCBS agar was not used) from 2 oysters.

   Following death of test oysters three more oysters were placed in that tank and observed. These oysters died from 24-27 days and *P. putrefaciens* was cultured from one. Three more oysters were placed in the tank and they died between 15 and 43 days. *P. putrefaciens* was again isolated from all oysters. There were no obvious histological changes.

2. During the course of investigations several causes of mortality have been suggested by people within the industry. Two of these were investigated.

2.1 Iron toxicity

   One operator suggested that rusty iron within carrying tanks was a cause of mortality. To investigate this several pieces of rusty iron were placed in an aquarium containing 5 oysters. There was no effect on the oysters over a 6 week period of observation.

2.2 Dead oysters as a source of infection

   It has been suggested by several people that if one oyster dies then oysters surrounding it will subsequently die. To test his hypothesis an oyster was refrigerated until moribund then placed in an aquarium with 4 normal oysters. The moribund oyster subsequently died, autolysed and dissolved but there was no effect on surrounding oysters. Within the laboratory individual oysters have commonly died but there has never been any indication of an effect on surrounding oysters.

Appendix 2

Histological findings in normal oysters

1. Intranuclear inclusion bodies

Basophilic or amphophilic intranuclear inclusions were commonly seen in the basiphil cells of the digestive gland tubules (Fig. 13) and less commonly in epithelial cells of the gut. The inclusions tended to be centrally located within nuclei and were surrounded by a clear zone. They stained purple with phloxine tartrazine, faint pink with methyl green pyronin, slightly basophilic with Feulgen stain, were acid fast negative and PAS negative. The inclusions measured 2.5 - 5.0 µm in diameter. Affected nuclei were 4.3 - 7.5 µm in diameter compared to unaffected nuclei which measured 2.5 - 3.0 µm.

Ultrastructurally the inclusions consisted of an electron-dense, finely granular matrix containing "doughnut-shaped" subunits surrounded by an electron-lucent halo (Fig. 14). The subunits measured 32.6 - 38 nm and the subunit and halo were 53.3 - 70.7 nm in diameter. Occasionally small inclusions were seen adjacent to the nuclear membrane. The sub-units resemble viral particles.

There were no other changes noted within affected cells.

2. Parasites

Sections of metazoan parasites surrounded by phagocytic cells were seen in the connective tissues of the visceral mass in a number of oysters. An apicomplexan protozoan parasite was commonly seen in the auricle (Fig. 15). This parasite consisted of vermiform cells 2.5 - 3.0 µm diameter, embedded in an eosinophilic matrix 25 - 55 µm diameter (Perkins, pers. comm.). One or several nuclei are present in each vermiform cell. The cells coiled around each other in the mass. Lesions were not seen in association with these parasites.

Large ciliated protozoa were common in the mid gut. The ciliates attached at their anterior end to the microvilli and cilia of gut epithelial cells (Fig. 16). They measured 10 - 11 x 30 - 41 µm. These organisms were not associated with disease but there was an apparent increase in their number in some diseased oysters.

3. Residual bodies in digestive gland epithelium.

In both normal and diseased oysters round, brownish, slightly refractile bodies have been seen in the apical cytoplasm of the digestive cells of the digestive tubules (Fig. 17). These structures were not apparent in the darker basiphil cells of the tubules. The round bodies were present in all oysters but the number varied considerably within normal and diseased oysters. The bodies measured 48 ± 1.2 µm. They failed to stain with

PAS reaction, toluidine blue, Perl's and Schmorl's stains. They did however have a red periphery when stained with saffranin. When sections were stained for acid phosphatase activity (Pearse, 1968) a reaction product was variably present over the round bodies.

Ultrastructually the round bodies were bound by a loose trilaminar membrane and contained a fibrillar and granular matrix (Fig. 18).

The light microscopic appearance, enzyme activity and ultrastructural appearance indicate that the round bodies are in fact "residual bodies" (Owen, 1970; 1972) which are either storage or excretary products within lysosomes. These structures are normal constituents of digestive cells.

Table 1. Physical and biochemical characters of *Vibrio* sp. isolated from morbid oysters.

	KB 9/81[a]	KB 7/82[a]	V. alginolyticus
Gram negative rod	+	+	+
Fermentative	+	+	+
Growth on SRBC agar[b]	poor	poor	poor
Motile	+	+	+
Growth in 0% NaCl	–	–	–
" " 3% "	+	+	+
" " 6% "	+	+	+
" " 8% "	+	–	
" " 10% "	–	–	–
Growth on TCBS agar	yellow	yellow	yellow
Swarming on fresh agar	+/–	–	+
ONPG	+	–	–
Arginine	–	–	–
Lysine	+	+	+
Ornithine	–	+	+
Citrate (Simmons)	+	+	–
H_2S	–	–	–
Urease	+	+	–
Tryptophane	–	–	–
Indole	+	+	+
Acetoin	–	–	+/–
Gelatin utilization	+	–	+
Acid from glucose	+	+	+
Mannitol	+	+	+
Inositol	–	–	–
Sorbitol	–	–	–
Rhamnose	–	–	–
Sucrose	+	+	+
Melibiose	–	–	–
Amygdalin	+	+	+
Arabinose	–	–	–
Lactose	–	–	–
Xylose	–	–	–
Maltose	+	+	+
Trehalose	+	+	+
Cellobiose	+/–	+	+
NO_3	+	+	+
Oxidase	+	+	+
Catalase	+	+	+
Antibiotic sensitivity:			
0/129	+		
chloramphenicol	+		
carbinicillin	+		
tetracycline	+		
trimethoprim/sulphasoxazole	+		
streptomycin	+		
ampicillin	–		
cephaloridin	–		

a) Identified as *Vibrio harveyi* by Dr R. Sakazaki National Institute of Health, Tokyo, Japan.

b) SRBC - sheep red blood cell agar.

TABLE 2. Experimental protocol.

Exp. No.	Bacterium	No. Oysters per group	Inoculum Mean cfu/ml	Inoculum Volume (ml) (a)	Method of Inoculation (b) MC	Method of Inoculation (b) EPS	Method of Inoculation (b) Tank (a)	Water Temperature 19°	Water Temperature 29°	Number of Inoculations Oyster	Number of Inoculations Tank
1	KB 9/81	5	7.4×10^8	4	+		+	+		4	4
2	KB 9/81	5	1.4×10^8	4	+				+	4	
3	KB 9/81	5	1.8×10^8	4		+			+	4	
4	KB 9/81	5	ND	4		+			+	1	
5	KB 9/81	5	1.7×10^8	2		+			+	4	
6	KB 9/81	5	- (c)	2		+			+	1	
7	KB 9/81	5	6.0×10^8	2		+		+	+	4	
8	KB 7/82	5	2.5×10^8	2		+		+	+	4	12
9	KB 7/82	6	4.7×10^8	2		+	+	+	+	4	4
10	KB 7/82	6	2.4×10^8	2		+	+	+		4	4
11	KB 7/82	13	7.0×10^8	2		+	+	+	+	4	4
12	*P. putrefaciens*	5	3.6×10^8	2		+			+	4	
13	*P. putrefaciens*	5	7.6×10^8	2		+	+	+	+	4	4

(a) Volume of Inoculum given to each oyster on each occasion. The remainder of the washed resuspended culture was added to the tank water where indicated. Control oysters received sterile saline as inoculum.

(b) MC - mantle cavity. EPS - extrapallial space i.e. between mantle and nacre.

(c) Culture contaminated with Bacillus sp.. Plate count unreliable.

N.B. On this and subsequent tables, the concentration of bacterial inoculum or growth is expressed as cfu's i.e., colony forming units.

Table 3. Experimental design of bacterial invasion experiment.

Day	0	1	2	3	4	5	6	7	8	15
Inoculate		+	+	+	+					
Bled	a		a			b			c	
Kill						b			c	d

a. Control and treated oysters bled

b. Controls only bled. (5 treated oysters killed)

c. Controls only bled (4 treated oysters killed)

d. 5 controls and 5 treated oysters killed.

Table 4. Design of bacterial toxinogenesis experiment.

GROUP	BACTERIUM	INOCULUM [a]			OYSTERS/ TREATMENT	MEAN cfu/ml ($x\ 10^8$)	CULTURE TIME HRS
		VIABLE CELLS	FREEZE THAWED CELLS	SONICATED CELLS			
1	KB 9/81	(4)			5	7.4	6
2	KB 9/81		(4)		4	ND	6
3	KB 7/82	(4)			5	6.0	6
4	KB 7/82			(4)	5	6.0	6
5	KB 7/82			(4)	5	10.0	18
6	P.putrefaciens	(4)			5	5.8	6
7	P.putrefaciens			(4)	5	5.8	6

[a] Numbers in parenthesis represent numbers of consecutive days on which Inoculum type was administered.

TABLE 5. Comparison of nutrients and chlolophyll a in Western Australian marine environments. Range of values are given for each sampling location.

LOCATION	PO_4-P (mg m^{-3})	Total - P (mg m^{-3})	NO_3- N (mg m^{-3})	NH_4- N (mg m^{-3})	Chlorophyll a (mg m^{-3})	SOURCE
COCKBURN SOUND	5 - 239	8 - 576	2 - 230	1 - 172	0.1 - 13.8	Chiffings 1979
OWEN ANCHORAGE	1 - 73	14 - 300	2 - 360	2 - 43	0.3 - 5.9	Chiffings 1979
GARDEN ISLAND COASTAL STN.	1 - 17	1 - 136	2 - 34	2 - 20	0.1 - 1.3	Chiffings 1979
WARNBRO SOUND	1 - 12	1 - 24	2 - 37	2 - 33	0.1 - 3.5	Chiffings 1979
MARMION COASTAL STN.	0 - 109	30 - 130	0.3 - 55			Johannes pers.comm.
ABROLHOS ISLANDS STN.	6 - 74	37 - 214	6 - 52	6 - 154	0.4 - 6.3	Hatcher pers.comm.
DAMPIER ARCHIPELAGO:						
MEMAID SOUND COASTAL STN.	1 - 9	15 - 86	2 - 13	1 - 16	0.1 - 1.7	Chiffings pers. comm.
	4 - 8	19 - 92	2 - 4	2 - 11	0.2 - 0.7	
EIGHTY MILE BEACH COASTAL STN.	3 - 7	17 - 62	3 - 6	1 - 13	0.1 - 1.4	This study
KURI BAY LEASE SITE	6 - 27	21 - 58	1 - 17	1 - 27	0.1 - 1.2	This study
OTHER LEASE SITES	3 - 10	21 - 43	2 - 7	3 - 13	0.1 - 3.0	This study

Table 6. Initial survival of pearl oysters after 42 days suspension.

SITE	SIZE SMALL NO. ALIVE	(%)	MEDIUM NO. ALIVE	(%)	LARGE NO. ALIVE	(%)
CAPE VILLARET	100		100		100	
LONGLINE	100	(100)	100	(100)	100	(100)
	100		100		100	
KURI BAY	46		83		93	
LONGLINE	53	(63)	85	(85)	100	(95)
	90		88		93	
KURI BAY RAFT	40	(40)	44	(44)	63	(63)

TABLE 7 . Number of surviving pearl oysters after a total of 144 days suspension.

MONTH OF TRANSFER	TEMP DIFFERENCE °C	DAYS ON LONGLINE	SIZE SMALL CV[a]	SIZE SMALL KB[b]	SIZE MEDIUM CV	SIZE MEDIUM KB	SIZE LARGE CV	SIZE LARGE KB
JULY	4.2	0[c]	-	16 -	-	15 -	-	41 -
AUGUST	2.5	42	31	28	28	44	50	83
SEPTEMBER	1.6	70	27	36	54	50	81	80
OCTOBER	1.4	99	100	60	100	63	100	80

a Pearl oysters transferred from the Cape Villaret long-line

b Pearl oysters transferred from the Kuri Bay long-line

c Pearl oysters placed directly on the Kuri Bay raft

TABLE 8. Mortality rate in relation to time and position during carriage.

Position	Trial 1		Trial 2	Trial 3	Trial 4
	% Mortality at 51 days		% Mortality at 54 days	% Mortality at 64 days	% Mortality at 38 days
	Day 1[a]	Day 3			
Bottom	49	36	31	80[b]	32[b]
Middle	44	31	40	73	22
Top	40	28	37	61	52
Bottom Horizontal	–	–	31	–	–
Top Horizontal	–	–	37	–	–

[a] Oysters collected on Day 1 and Day 3 of the neap tide.

[b] Significant difference ($p < 0.01$) between the bottom and top.

TABLE 9. Mean and standard deviation (SD) of *Vibrio* sp./ml of surface water and the ratio of *Vibrio* sp. in water of carrier tanks to that in seawater (TANK:SW).

Day	Morning Mean ± SD		Morning Tank:SW[b]	Evening Mean ± SD		Evening Tank:SW
			Transport vessel A July 1982. Full load[a].			
1	10[c] ±	0.9	1.3	23 ±	3.5	2.9
2	179	-	22.9	500 ±	192	64.1
3	960	-	123.1	978 ±	111	125.4
4	368 ±	69	47.2	N D[d]		
5	46 ±	14	5.9	1 725[e] ±	357	221.2
				20 138[f] ±	6 504	2 581.8
			Transport vessel B July 1982. Half-load.			
1	3.4 ±	1.5	0.6	6.3 ±	3.2	1.1
2	59.4 ±	18.2	10.4	453.9 ±	237.1	79.6
3	182.5 ±	87.7	32.0	408.6 ±	67.9	71.7
4	29.3 ±	19.7	5.1	172.5 ±	48.4	30.3
5	85.0 ±	21.9	14.9	5 573.3[e] ±	232.9	977.8
				10 020.0[f] ±	2 120.0	1 757.9
			Transport vessel B April, 1983. Half-load.			
1	195.0[c] ±	92	1.7			
1	158.0[g] ±	35	1.4	674 ±	81	5.9
2	6 123.0 ±	572	53.7	7 683 ±	1 009	67.4
3	2 193.0 ±	302	19.2	363 ±	107	3.2
4	198.0 ±	44	1.7			
	1 012.0[e] ±	116	8.9			
	19 093.0[f] ±	7 196	167.5			

a Refers to full or half load of oysters.

b Ratio of number of bacteria in tankwater to seawater.

c Before tanks were loaded with shell.

d Not Done.

e After tanks were unloaded at lease.

f Bottom sludge after tanks pumped out.

g After tanks were partially loaded with shell.

Table 10. Morbidity/Mortality data for transmission experiments.

Exp. No.	Temp °C	Inoculum	Morbidity No.affected/No.tested (%)		Days until (Range) Morbid		Days until (Range) Dead		No. that died		Bacteria in haemolymph		Lesions[e]	
			Test	Control	Test	Control	Test	Control[a]	Test	Control	Test	Control	Test	Control
1	19	KB 9/81	5/5 (100)	1/5 (20)	4-18	21	11-21	21	5	1	4/5	1/5	3/5	1/5
2	29	KB 9/81	5/5 (100)	2/5 (40)	15-23	17-22	28	28	0	0	0/5	0/5	1/5	2/5
3	29	KB 9/81	4/5 (80)	0/5 (0)	3-11	-	3-11	28	3	0	2/4	0/5	2/4	0/5
4	29	KG 9/81	1/5 (20)	0/5 (0)	19	-	19[b]	28	0	-	0/5	0/5	1/5	0/5
5	29	KB 9/81	2/5 (40)	1/5 (20)	13-32	11	35	35	0	0	1/5	2/5	1/5	0/5
6[d]	29	KB 9/81	1/5 (20)	1/5	5	-	12/33	-	1	-	1/5	2/5	ND	0/5
7	29	KB 9/81	0/5 (0)	0/5 (0)	-	1-7	39	39	0	0	0/5	2/5	ND	ND
	19[c]	KB 9/81	2/5 (40)	5/5 (100)	8-34	1-15	35-41	8-27	0	2	4/5	4/5	ND	ND
8	29	KB 7/82	1/5 (20)	0/5 (0)	1-18	-	35	35	0	0	3/5	2/5	ND	ND
	19	KB 7/82	1/5 (20)	0/5 (0)	8	-	35	35	0	0	4/5	1/5	ND	ND
9	19	KB 7/82	5/6 (83)	1/6 (17)	13-30	16	15-36	26-36	4	1	5/6	1/6	ND	ND
10[d]	19	KB 7/82	5/6 (83)	-	4-8	-	14-25	-	4	-	5/6	1/6	ND	-
11	19	KB 7/82	11/13 (85)	7/18 (54)	8-22	5	13-27	12-31	4	3	4/13	9/13	ND	ND
	29	KB 7/82	1/13 (8)	0/13 (0)	8	0	23-31	23-31	0	0	0/13	0/13		
12[d]	19	P. putrefaciens	4/5 (80)	-	12-24	-	22-36	-	3	-	3/5	-	ND	-
13	29	P. putrefaciens	5/5 (100)	0/5	3-18	-	4/29	29	1	0	5/5	0/5	1/5	0/5

(a) The highest figure indicates the day on which the experiment was terminated. In experiments 9,10 and 11 all remaining live oysters were killed between the days indicated for the control group.

(b) All oysters killed. Controls for experiment 4 were those used in experiment 3.

(c) See text for explanation.

(d) Controls for experiment 6 were those used in experiment 5 and controls for experiments 10 and 12 were those used in experiment 9.

(e) Lesions were the same as those described in the natural disease.

TABLE 11. Morbidity and mortality data for oysters held at 19° and 29°.[a]

	Infected with Vibrio sp.		Control	
	19°	29°	19°	29°
Morbidity	32/40 (80.0%)	16/38 (42.1%)	15/34 (44.1%)	4/33 (12.1%)
Mortality	17/40 (42.5%)	4/38 (10.5%)	8/34 (23.5%)	0/33 (0%)

[a] Number affected/number tested

TABLE 12. Number and (%) of oysters in which *Vibrio* sp. were isolated from haemolymph and muscle.

	ISOLATION OF BACTERIA IN:					
	Haemolymph					Muscle
	NG[a]	Slight	Moderate	Heavy	V.heavy	
Pre-inoculation	16/20 (80)	4/20 (20)				
2 DPI[b]-treated	1/15 (7)	2/15 (13)	9/15 (60)	2/15 (13)	1/15 (7)	
control	4/5 (80)	1/5 (20)				
4 DPI-treated	1/5 (7)	1/15 (7)	9/15 (60)	2/15 (13)	2/15 (13)	
control		1/5 (20)	3/5 (60)		1/5 (20)	
5 DPI-treated			1/6 (17)	3/6 (50)	2/6 (33)	5/5 (100)
5 DPI-treated			1/5 (20)	1/5 (20)	3/5 (60)	5/5 (100)
16 DPI-treated					4/4 (100)	4/4 (100)
control			3/5 (60)	1/5 (20)	1/5 (20)	2/5 (40)

a NG - No growth, slight (1-20 colony forming units), moderate (21-100 cfu's), heavy (101-300 cfu's), v. heavy (> 300 cfu's).

b DPI - days post-inoculation.

Table 13. Effect of culture filtrates on oysters[a]

	Control		Treated	
Group	Diseased	Dead	Diseased	Dead
1	4/5(80)	2/5(40)	3/5(60)	3/5(60)
2	3/4(75)	3/4(75)	4/4(100)	4/4(100)
3	0/5(0)	0/5(0)	5/5(100)	5/5(100)
4	ND	ND	5/5(100)	5/5(100)
5	ND	ND	5/5(100)	5/5(100)
6	ND	ND	4/5(80)	2/5(40)
7	ND	ND	4/5(80)	0/5(0)

Groups 1-5 inoculated with *V.harveyi*.
Groups 6 and 7 inoculated with *P.putrefaciens*.

a Number affected/number tested (%)

ND = not done

Table 14. Mean ± SD for Condition Index

Experiment	Temperature			
	19°		29°	
	Control	Treated	Control	Treated
7	12.3±2.6	11.8±1.2	8.7±0.5	10.5±1.5
8	9.7±0.8	11.0±0.9	9.9±.0.8	10.0±0.7
11	13.2±1.3	12.9±2.4	10.9±1.4	10.0±0.7

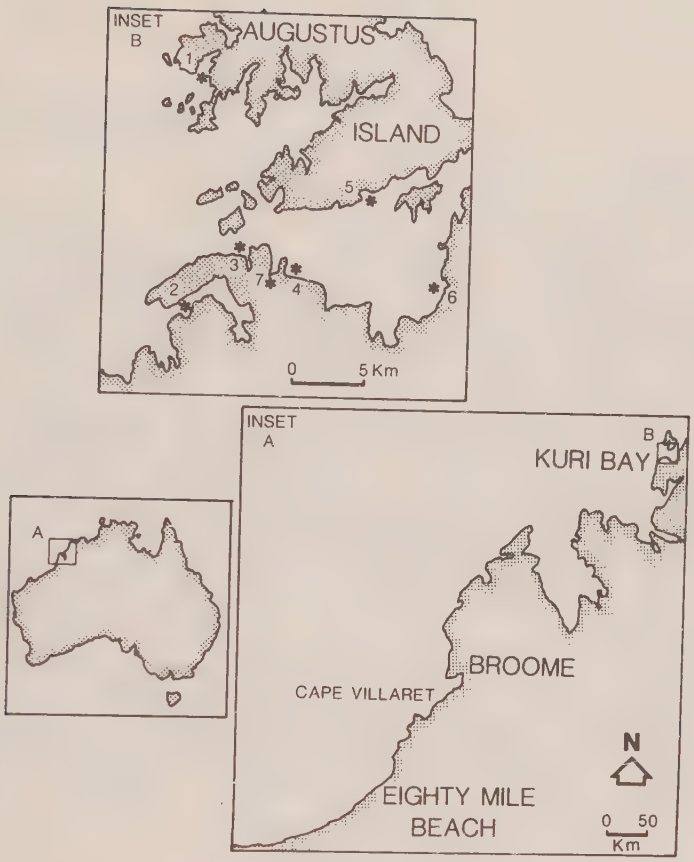

Figure 1. Location diagram of Northwest Australia showing main pearling areas referred to in the text. Study sites at Kuri Bay (Inset B) are as follows: (1) Hiro Bay, (2) Sampson, (3) Kuri Bay B Place, (4) Kuri Bay C Place, (5) S-Place, (6) Camden B and (7) Kuri Bay A Place and Settlement.

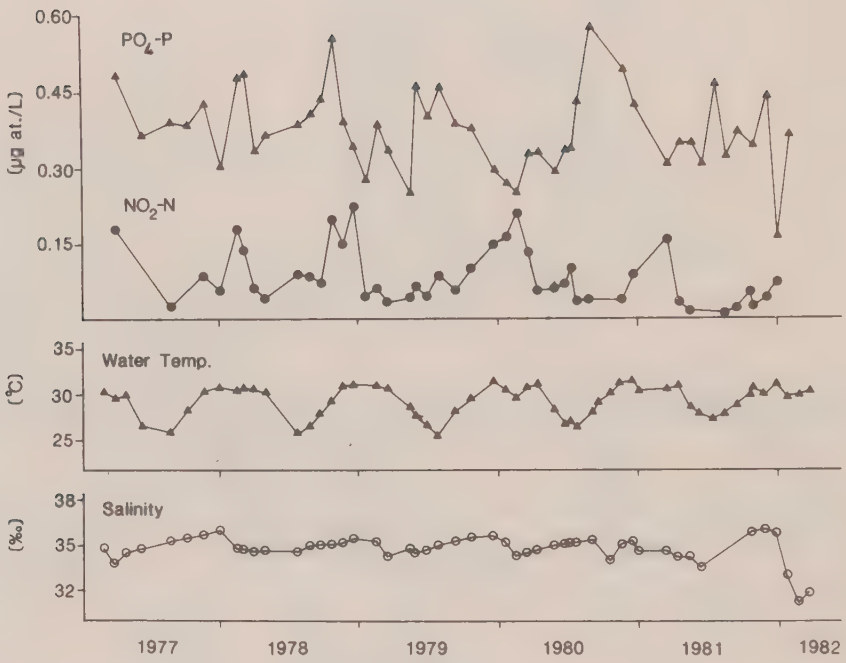

Figure 2 . Time series of industry records of four water quality parameters sampled at the Kuri Bay C Place between March 1977 and April 1982.

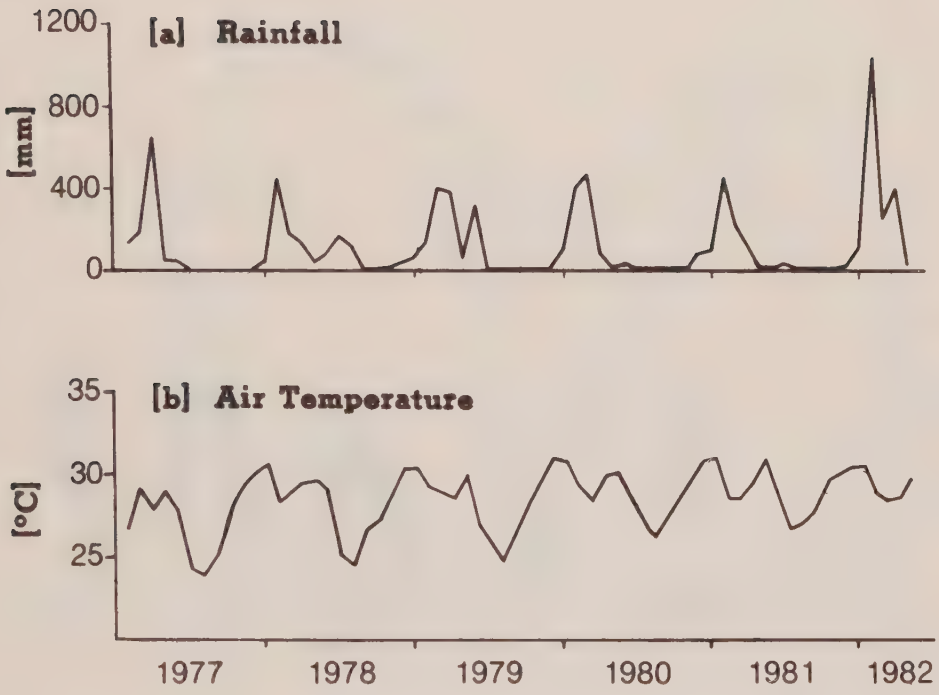

Figure 3. Time series of Department of Meteorology's monthly records of total rainfall (A) and average air temperature (B) for Kuri Bay between January 1977 and April 1982.

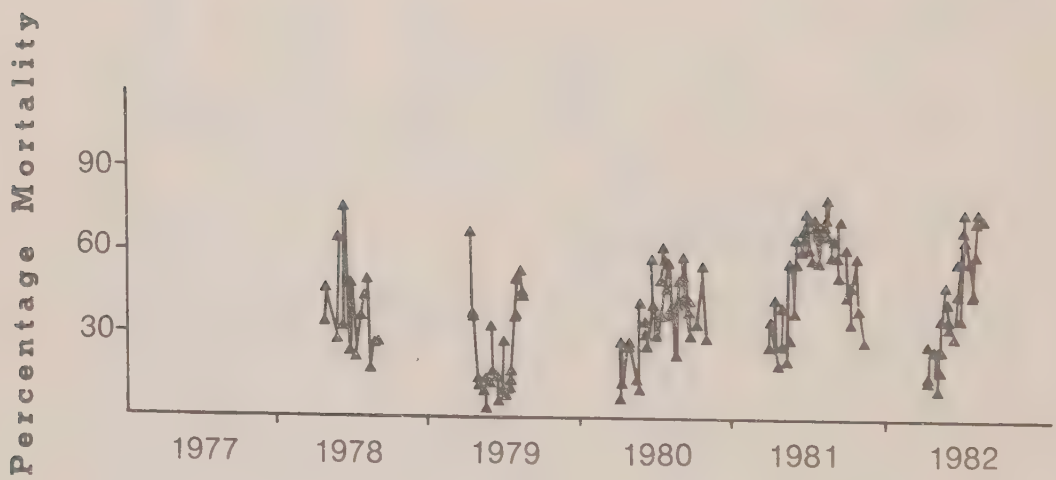

Figure 4. Time series of industry records of percentage mortality of pearl oysters for individual collection trips for the pearling seasons between 1978 and 1982.

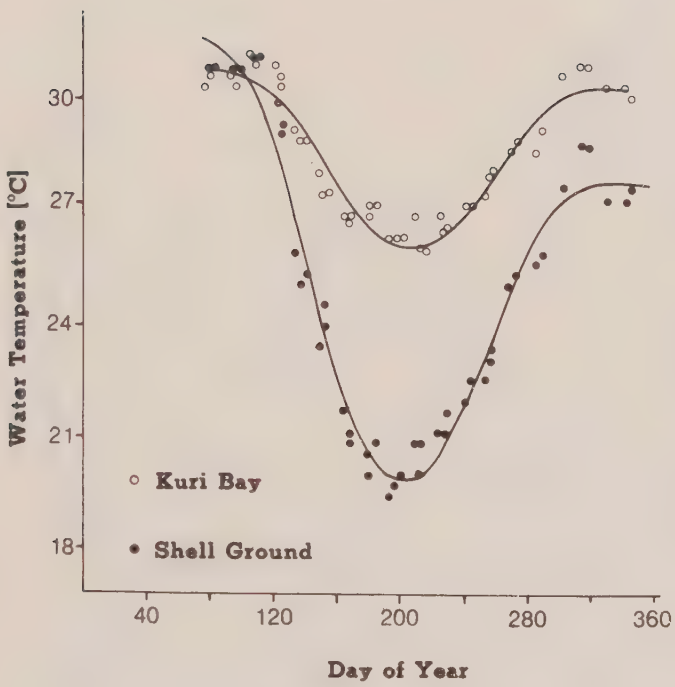

Figure 5. Surface sea water temperatures at Kuri Bay and the shell grounds over the 1981 pearling season. Fitted cyclical curves where $\theta = 2\pi$ (day of the year)/ 365 gave the following equations:

$$T_{KB} = 29.09 + 0.85 \sin(\theta) + 1.59 \cos(\theta) - 0.65 \sin(2\theta) - 0.79 \cos(2\theta)$$

with correlation (r) = 0.97, sample size (N)=155; and

$$T_{SG} + 26.15 + 2.74 \sin(\theta) + 3.85 \cos(\theta) - 0.63 \sin(2\theta) - 2.10 \cos(2\theta)$$

with r = 0.98, N = 126.

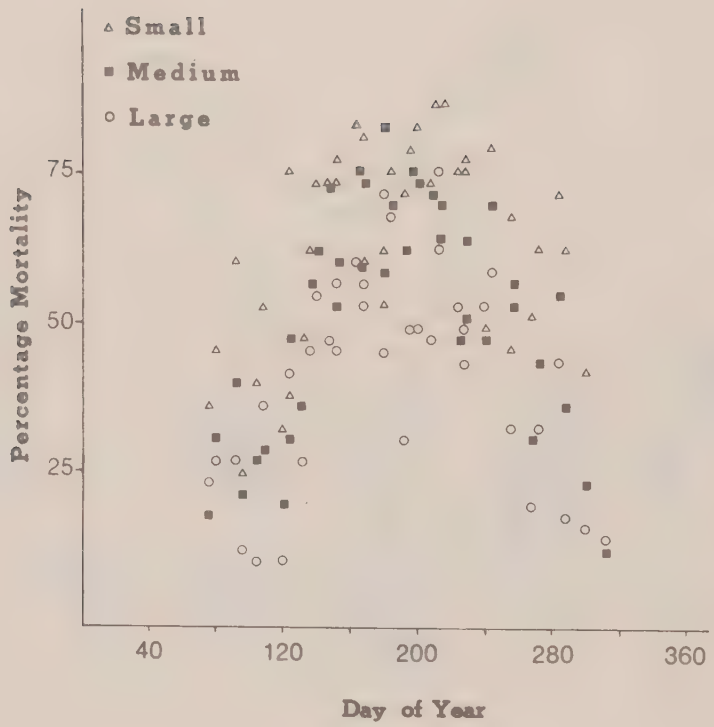

Figure 6. Percentage mortality of pearl oysters of three size categories from individual collection trip records over the 1981 pearling season.

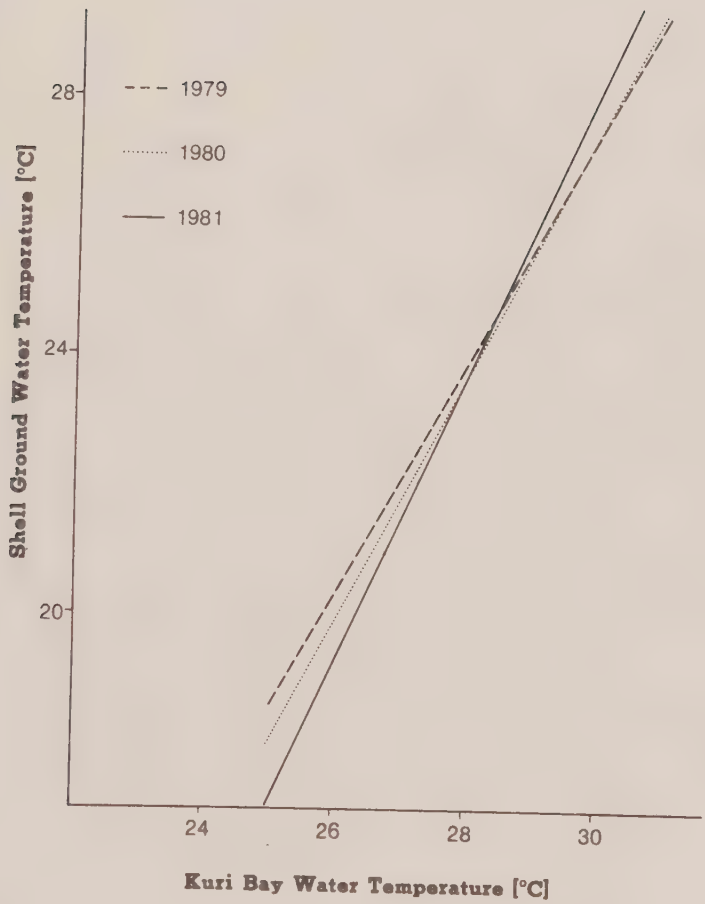

Figure 7. Linear regressions of surface water temperatures between the shell grounds and the Kuri Bay lease site. For the 1979 pearling season,

$T_{SG} = -25.60 + 1.766\ T_{KB}$ with $r = 0.94$, $N=32$.

For 1980, $T_{SG} = -28.64 + 1.864\ T_{KB}$ with $r = 0.94$, $N=35$.

For 1981, $T_{SG} = -38.00 + 2.198\ T_{KB}$ with $r = 0.97$, $N=45$.

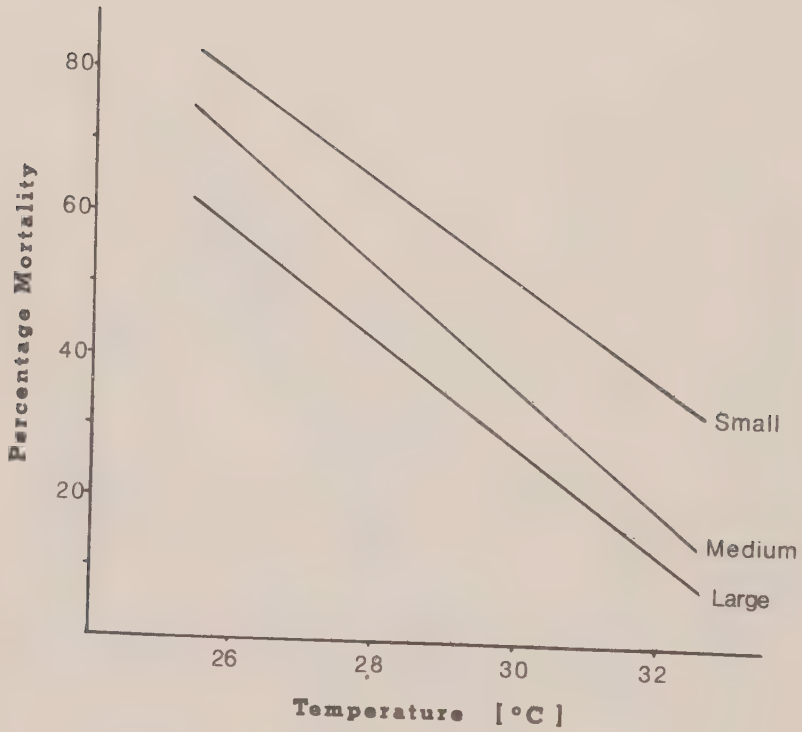

Figure 8. Relationship between percentage mortality of pearl oysters of three size categories following transport to the Kuri Bay lease site and the water temperature at this lease upon arrival over the 1961 pearling season. Regression lines are derived from temperature data given in Figure 5 and percentage mortality data given in Figure 6.

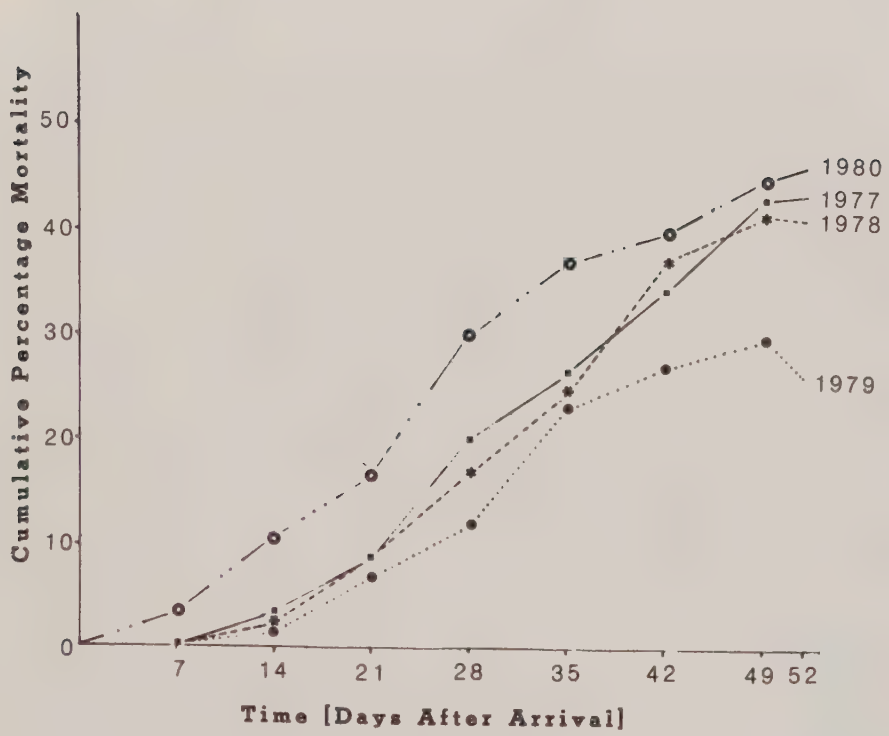

Figure 9. Mean cumulative percentage mortality of oysters after arrival at the lease site using all collection trip records for each fishing season for the years 1977-80.

Figure 10. Accumulation of large numbers of phagocytes (A) in the connective tissue septa of the digestive gland. Hematoxylin and eosin stain.

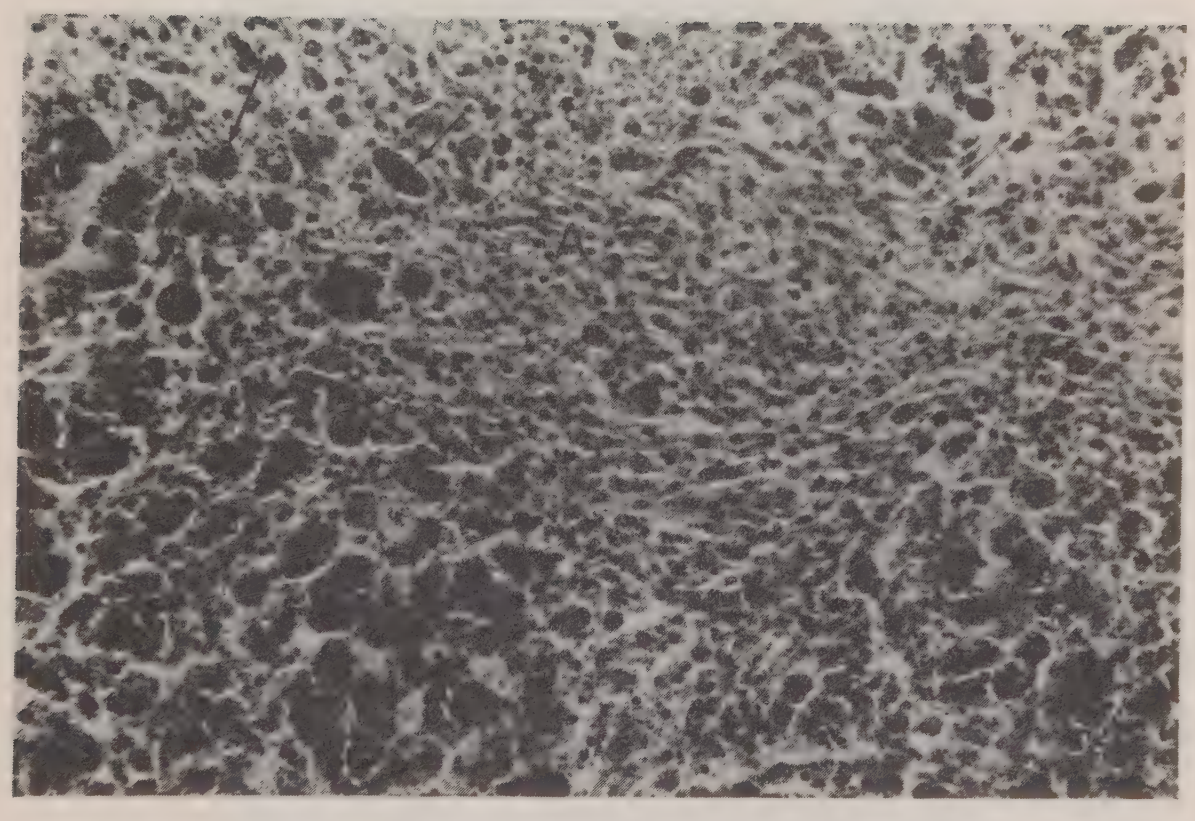

Figure 11. Accumulation of fibrous like phagocytes (A) in the auricle. The granular cells (arrows) are "brown cells" that are normally present in the auricle. H E.

Figure 12. Section through mantle tissue showing erosion and phagocyte exudation (arrow) of the epithelium of the outer fold (A) of the mantle. H E.

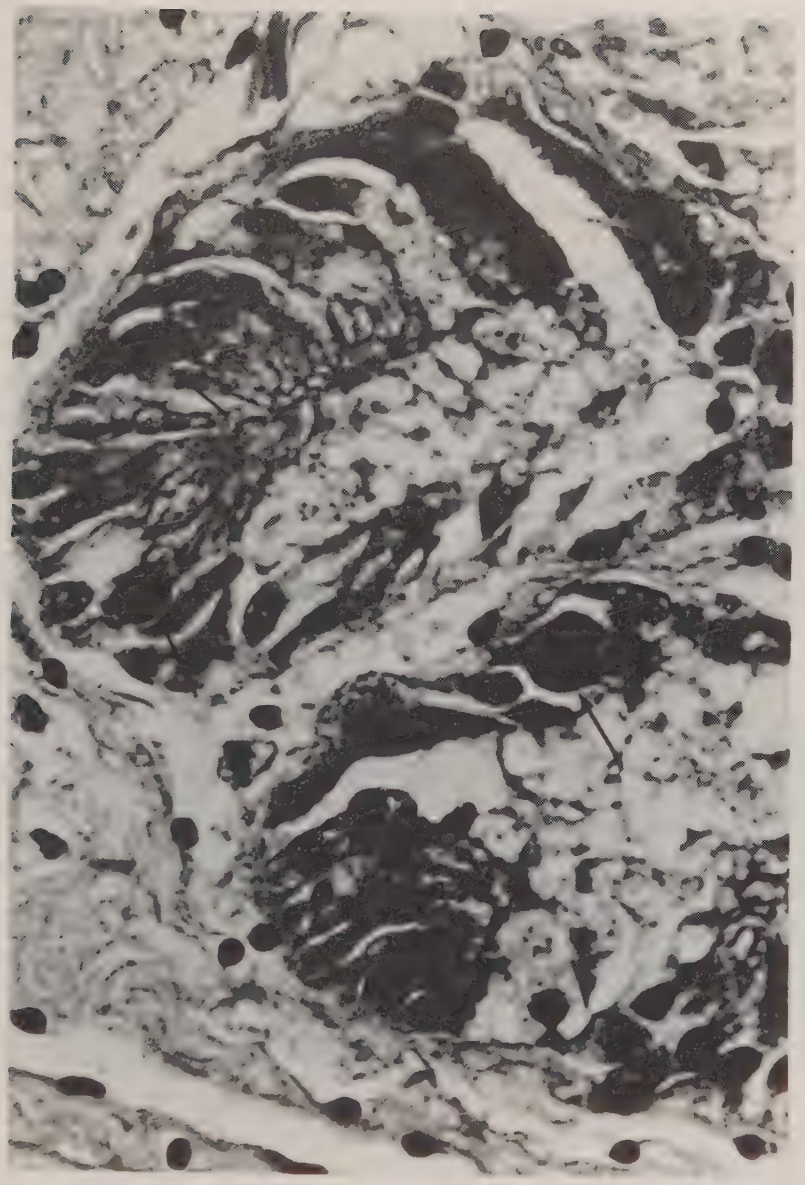

Figure 13. Digestive gland. Dense intranuclear inclusion bodies (arrows) in basiphil cells of digestive tubules. H E.

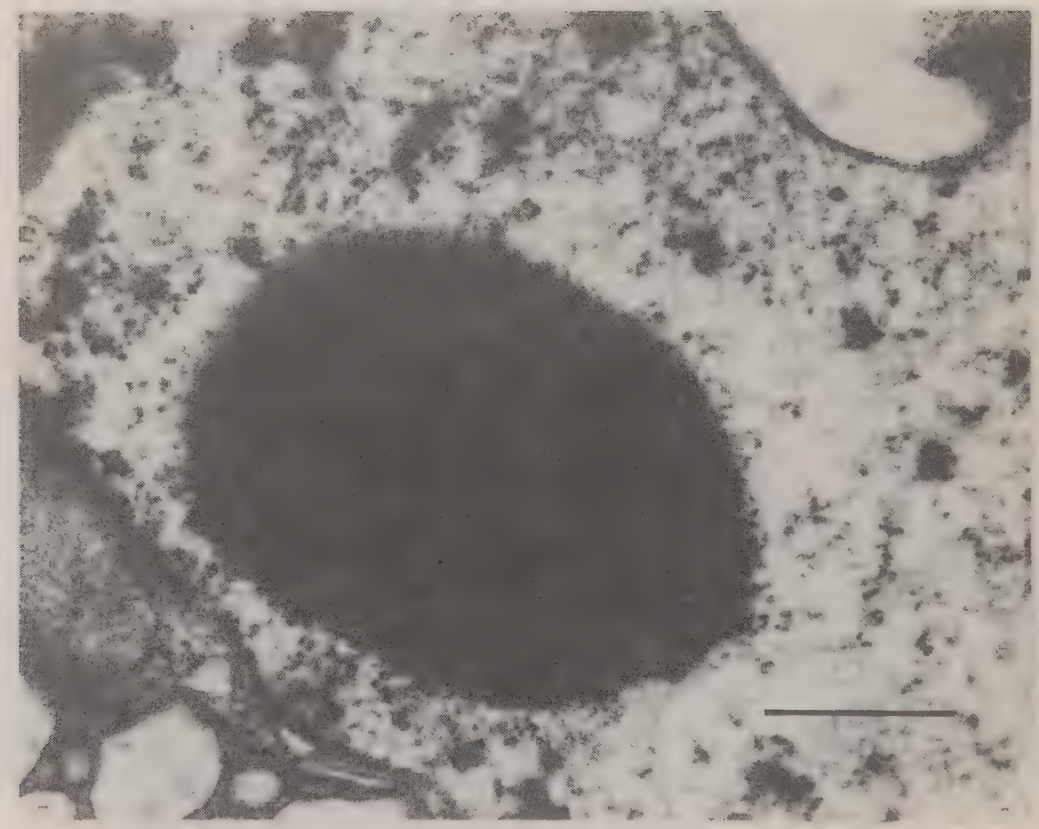

Figure 14. Electron micrograph of intranuclear inclusion body containing virus-like particles. Bar indicates 1 μm.

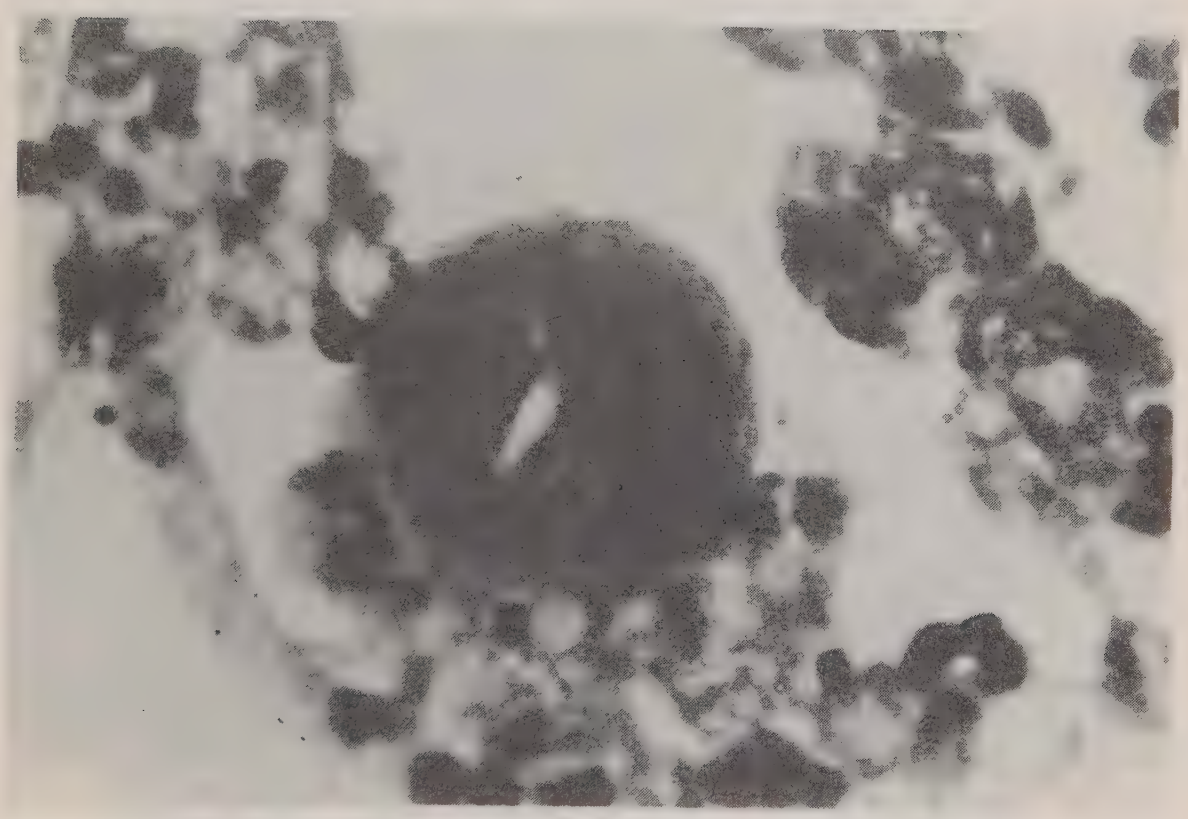

Figure 15. Apicomplexan protozoan in auricle. H E.

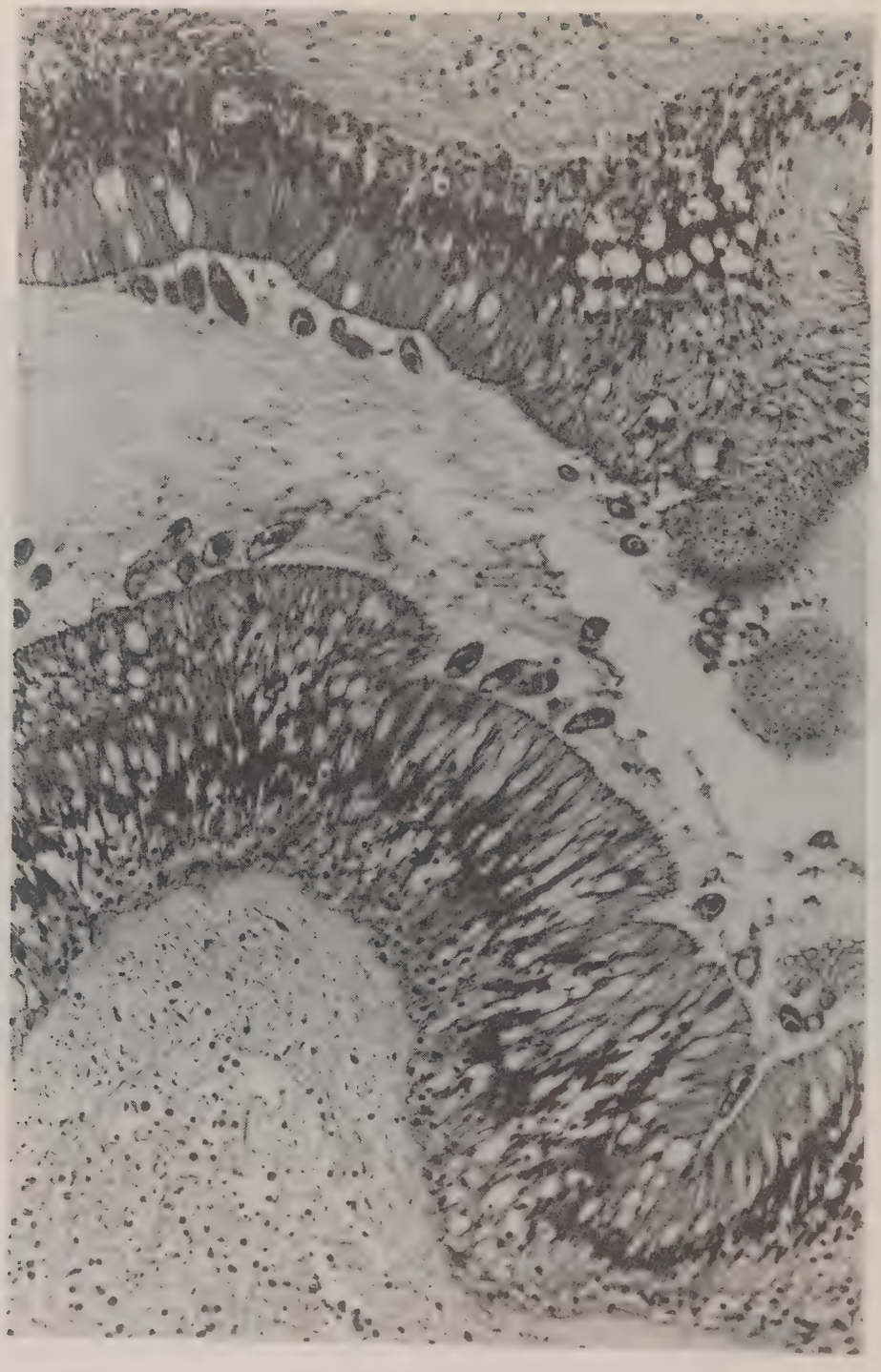

Figure 16. Ciliated protozoa attached to the mid-gut epithelium. H E.

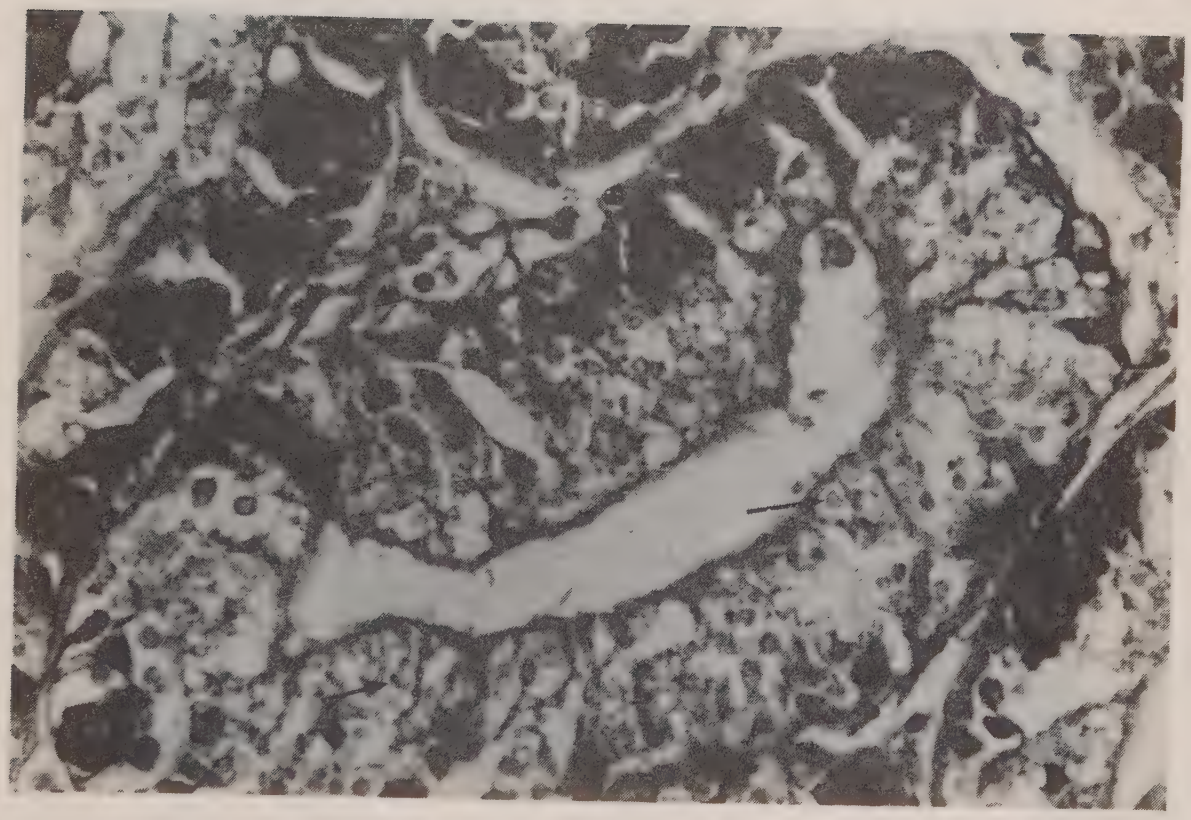

Figure 17. Residual bodies (arrows) in the cytoplasm of digestive cells of digestive gland tubules. H E.

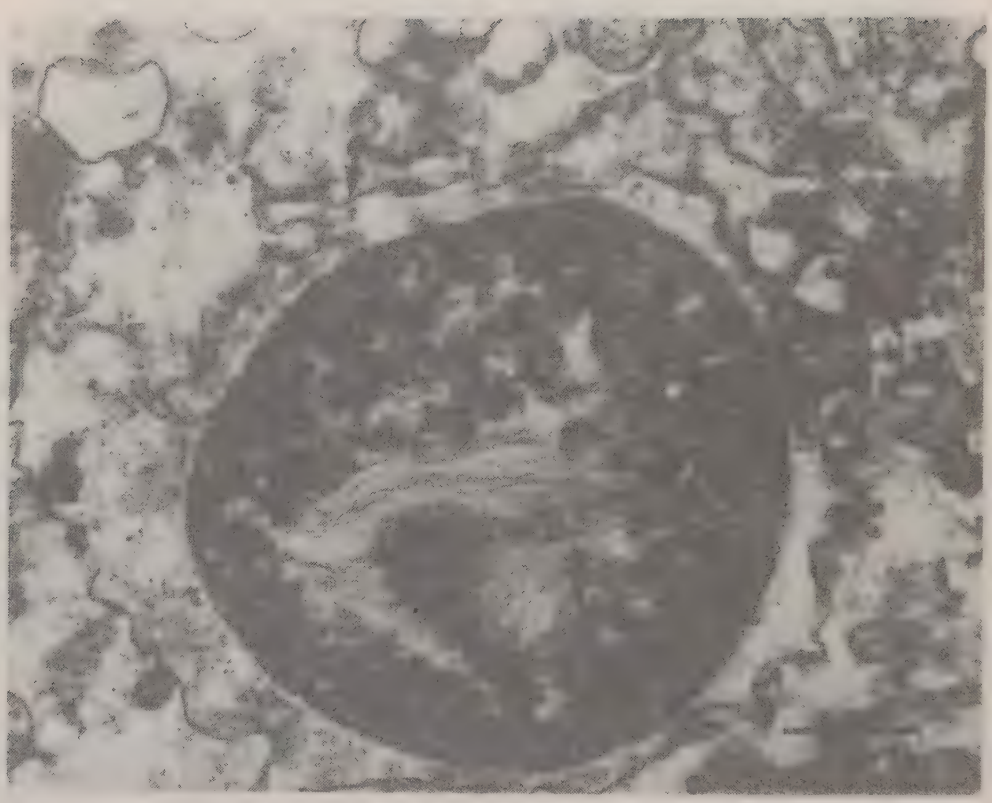

Figure 18. Electron micrograph of a residual body. Lysosomal membrane (arrow). Bar indicates 1 μm.

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